ASSESSING THE DEVELOPMENT AND DETECTION OF BIOGENIC AMINES AS INDICES FOR AQUATIC PRODUCT QUALITY AND SAFETY

By

WEN-XIAN DU

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Dedicated to my dear MoM & DaD

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Wen-Xian Du

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Refrigeration time and temperature during processing and distribution affect the quality and safety of seafood products. Microbiological assessment, biogenic amine determination, and sensory analysis were performed on fresh salmon, mahi-mahi, and tuna stored at different temperatures for various time periods. Color, texture, appearance, and odor were evaluated by a trained sensory panel, while aroma/odor was evaluated using an AromaScan. Bacterial enumeration was performed using aerobic plate count (APC) agar containing 1.5% NaCl and incubated for 48 hr at room temperature. Histamine-producing bacteria were isolated from salmon and tuna fillets using Niven's differential medium and identified by API 20E strips. Histamine was determined using capillary electrophoresis (CE) and AOAC fluorometric method. Biogenic amines were determined using a gas chromatograph (GC) after derivatization. The objectives of this study were (1) to correlate

the degree of agreement among bacterial numbers, sensory analysis, biogenic amine levels, and AromaScan evaluation for quality determination of salmon, mahi-mahi, and tuna; and (2) to determine the applicability of these methods for quality determination of the above-tested seafood.

Storage temperature and microbial counts played important roles in the spoilage of fresh seafood. The change in quality correlated with increases in microbiological measurements. Monitoring bacterial levels is a useful objective indicator of gross spoilage and the presence of histamine hazard in mahi-mahi and tuna.

Histamine level has little value in monitoring the loss of freshness that occurs in fish fillets during storage at different temperatures. Biogenic amines may be used as quality and safety indicators for mahi-mahi and tuna. Very good correlations ($r^2 > 0.99$) were found among AOAC, CE, and GC methods for determination of histamine in tested mahi-mahi and tuna samples. The CE method may offer an alternative procedure for rapid screening and determination of histamine in seafood. The GC procedure for simultaneous analysis of 5 putrefactive amines provides a useful tool for assessing these chemical indicators in spoiled seafoods.

Human sensory analysis provides a useful means to monitor both changes in freshness and the onset of spoilage. In determination of seafood quality, good correlation exists between AromaScan and sensory analysis, and AromaScan and bacterial counts. AromaScan analysis may provide a viable, quantitative approach to determine fish freshness which could be used for quality control and inspection purposes.

CHAPTER 1
INTRODUCTION

The U. S. seafood and aquaculture industries are experiencing dramatic changes in all aspects of production, commerce, regulation, and public perception. These changes are evidenced by an increasing per capita consumption and production that cannot meet consumers' demand. The value of U. S. seafood imports increased from $6.8 billion in 1996 to $7.8 billion in 1997 (NMFS, 1997). Challenges for more health-conscious products and consumers who expect higher product quality and safety are also putting increased pressure on seafood processors. International seafood commerce is challenged by emerging markets and elevated quality standards. With the current global predictions of a shortfall of 10 to 40 million metric tons in traditional aquatic food productivity by the year 2010, aquaculture is expected to play an important role in offering new resources for supply (FAO, 1998). New products from aquaculture and importation will introduce unique concerns. Also, new processing innovations catering to convenience and value-added concepts have introduced new food safety concerns.

Seafoods are highly susceptible to spoilage and deterioration due to autolysis and the growth of postmortem microbial populations (Dainty et al., 1983). Microbial activity on seafood products produces pronounced off-flavors and off-odors, a shorter shelf life, and heavy economic losses (Reddy et al., 1994).

Freshness and spoilage are the principal terms used to describe quality changes in seafood products. Final product quality is affected by handling, gutting, and storage temperatures. Although refrigeration and freeze storage can extend the shelf life of seafood products, proliferation of psychrotrophic bacteria at refrigerated temperatures still contributes to their spoilage (Hobbs, 1983).

While spoilage is an important criterion for determining the overall quality of seafood products, considerable effort has been expended to search for suitable methods to assess freshness while the product is still edible (Pivarnik et al., 1990). Traditionally, assessment of seafood freshness has relied on sensory evaluation methods. Taste panels are used in the seafood industry to classify products as fresh or spoiled. Although fast, simple and, for the most part, accurate, sensory analysis is sometimes perceived to be inherently subjective. The taste panel, no matter how skilled, is not oncall during all phases of production, nor can it function efficiently for prolonged periods of time. Although chemical methods using chromatographic and wet chemical techniques for determining seafood freshness are precise and objective, they are time-consuming and expensive and are species dependent. Laboratory space is required and analyses must be performed by technically qualified personnel. Therefore, there is a need to develop new technologies that can give an instantaneous and objective classification of seafood quality and freshness so the processing industry can quickly release and maintain the quality of their products.

Of all the senses, smell has been the most difficult to define objectively. Only in the last several years has the ability to measure and characterize smell become possible. Advances in organic chemistry, electronics, and computing in recent years have combined

to make possible the development of new digital aroma technology (electronic nose) which may parallel the human nose (Russell, 1995). The potential for the electronic nose is vast. It is anticipated that there will be a considerable reduction in quality-control costs when the nose is used to replace expensive product-tasting panels for routine quality analysis.

AromaScan (AromaScan Inc., Hollis, NH) is a highly sophisticated electronic nose unit which mimics the human nose, and can be used for objective classification of product quality. As aroma/odor vapors from samples being analyzed are drawn over an array of 32 electrically conducting organic polymer sensors, the volatiles are adsorbed on the polymer surface and then rapidly desorbed, thus causing temporary changes in electrical resistance. Data obtained from each sensor can be expressed as an aroma histogram using the AromaScan's software. The combined sensor values collected from the 32 sensors represent the continuous and quantitative real-time analysis of the overall aroma/odor for each sample, which can be condensed into a single representative data point on a two-dimensional AromaMap. The axes representing Euclidean distances between each sample can be plotted and the differences among samples determined. Multiple samples appear as populations on the AromaMap and demonstrate the reproducibility of using AromaScan for quality determination (Russell, 1995). Therefore, AromaScan can be used in parallel with sensory panels or to complement or replace more costly and time-consuming chemical analysis techniques such as gas chromatography/mass spectroscopy. With an AromaScan, samples need very little preparation time and so the analysis can be completed within a few minutes.

Although the electronic nose has been applied in grading coffee, detecting the adulteration of whiskey and wine, classifying grains and fresh fish, controlling beer

fermentation, and differentiating the quality of aged Parmesan cheese (Josson et al., 1997; Russel, 1995; Rogers et al., 1995; Alpha M.O.S., 1995), AromaScan has not been widely tested with seafood products. Research conducted by AromaScan, Inc., demonstrated that the AromaScan analyzer can clearly discriminate among four different classes (fresh, early decomposed, very decomposed, and rancid) of freshness in mahi-mahi and scallops.

For the seafood industry, monitoring of chemicals associated with seafood safety is as important as quality assurance. Scombrotoxic fish poisoning is associated with the consumption of spoiled fish from the families *Scomberesocidae*, *Scombridae,* and other non-scombroid fish such as bluefish, skipjack, tuna, mahi-mahi, bonito, amberjack, herring, mackerel, sardines, anchovies, etc., containing hazardous levels of histamine in muscle tissue (Arnold and Brown, 1978; Behling and Taylor, 1982; Roberts et al., 1995). Toxicity is associated with bacterial spoilage where bacteria break down histidine to histamine and possibly other toxic end products. Histamine toxicity in guinea pigs was potentiated following administration of some diamines including cadaverine (Bjeldanes et al., 1978). Therefore, other biogenic amine levels may also be important for histamine toxicity. Scombroid poisoning is one of the major food-borne illnesses reported to the Centers for Disease Control and Prevention in the United States and Britain (Bean and Griffin, 1991; Morrow et al., 1991; Taylor et al., 1989). The Food and Drug Administration (FDA) established a defect action level of 50 ppm for histamine in tuna, mahi-mahi, and other fish species as an indication of potential health hazards (FDA, 1996). Estimation of the number of spoilage organisms present is not a reliable indicator of possible toxicity, and these toxic substance(s) are not destroyed by cooking or curing the fish. The presence of histamine in

fish leads to health problems for consumers, therefore, it is important to monitor the level of histamine in fish during storage.

Salmon sold in Florida are usually aquacultured and shipped from Chile. Depending on shipping time and storage conditions, the salmon may have begun to decompose. Decomposition of aquatic food products occurs right after harvesting through the action of bacteria, endogenous enzymes, or abusive handling. Rapid and reliable methods for predicting decomposition would be very valuable to seafood processors. Histamine production is usually associated with scombroid poisoning from scombroid fishes (such as tuna). Salmon and mahi-mahi are not considered in this category of fishes. However, recent interest has focused on histamine and other biogenic amines as indicators of product decomposition. The Hazard Analysis and Critical Control Point (HACCP) implementation mandates specifically the monitoring for these safety indicators, yet routine procedures are lacking. In addition, information on using biogenic amine indicators to assess quality for most seafoods under actual commercial conditions is limited. Very little work has gone into establishing whether biogenic amine formation relates to product quality. The seafood industry and regulatory agencies need this information to evaluate current guidelines for histamine in seafood and to establish new regulatory guidelines, including action levels for biogenic amines (cadaverine and putrescine) as indices of aquatic product quality and safety. Therefore, faster, more accurate routine methods, such as AromaScan, that can be used at commercial landings or in the field for screening inferior products before purchasing will be beneficial to processors and regulators and will help reduce potential risk to consumers.

The objectives of this study are: (1) to determine biogenic amine levels in salmon, mahi-mahi, and tuna using capillary electrophoresis, gas chromatography, and AOAC fluorometric methods at various storage times (0, 1, 3, 5, 7, 9, 14 days) and temperatures (1.7°, 7.2°, and 12.8°C or -20°, 0°, 4°, 10°, and 22°C); (2) to perform quality evaluations (odor, texture, color, and appearance) on the above-tested seafood by a trained sensory panel and an AromaScan; (3) to determine the quality of the tested seafood samples using microbial counts; (4) to determine by statistical analyses of the data obtained from objectives 1-3 the accuracy of the hypothesis that biogenic amines (histamine, cadaverine, and putrescine) can be used as chemical indices for aquatic food quality and safety; (5) to ascertain the degree of agreement between AromaScan analyses and organoleptic assessment; and (6) to determine the feasibility and reliability of using an instrumental procedure based on AromaScan analysis for seafood quality evaluation which would have the accuracy, precision, and objectivity of the chemical methods, yet be faster and simpler for seafood quality assessment.

CHAPTER 2
LITERATURE REVIEW

Biogenic Amines in Fish and Other Food Products

Biogenic Amines

Biogenic amines are defined as basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Maijala et al., 1993). They are organic bases of low molecular weight and are synthesized from microbial, vegetable, and animal metabolisms. Biogenic amines in food and beverages are formed by the enzymes of raw material or are generated by microbial decarboxylation of amino acids (Brink et al., 1990; Halász et al., 1994). However, it has been found that some of the aliphatic amines can be formed *in vivo* by amination from corresponding aldehydes (Maijala et al., 1993).

Polyamines exist widely in biological materials and also in many food materials. Most are produced by enzymatic degradation associated with putrefaction or fermentation in foods, and they show physiological activity in humans. Amine levels were shown to be a good index of food decomposition (Mietz and Karmas, 1977). The polyamines usually do not cause any health concerns to individuals unless large amounts are ingested. However, some of the polyamines may be nitrosated in the presence of nitrite or act as precursors for other compounds capable of forming carcinogenic nitrosamines. On the other hand, some

of the polyamines are also known to potentiate histamine toxicity and act synergistically in allergy-like food poisoning caused by the intake of histamine-contaminated food.

The chemical structure of biogenic amines can either be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine), or heterocyclic (histamine, tryptamine). Amines, such as polyamines, putrescine, spermidine, spermine, and cadaverine, are indispensable components of living cells. They are important in the regulation of nucleic acid function and protein synthesis and perhaps also in the stabilization of membranes (Bardócz et al., 1993; Maijala et al., 1993; Halász et al., 1994). The diamines putrescine and cadaverine belong to a group of natural polyamines which serve as stabilizing cations for the macromolecular structures of DNA and RNA; they can be expected to be abundant in all living matter (Cohen, 1978).

The structures of the main biogenic amines and their precursors are shown in Figure 1. Prerequisites for biogenic amine formation by microorganisms are: (1) availability of free amino acids (Marklinder and Lönner, 1992), (2) presence of decarboxylase-positive microorganisms, and (3) conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Brink et al., 1990).

Biogenic Amines in Food

Biogenic amines can be expected in virtually all foods that contain proteins or free amino acids and are subject to conditions enabling microbial or biochemical activity. The total amount of the different amines formed strongly depends on the nature of the food and the microorganisms present. Biogenic amines are present in a wide range of food products

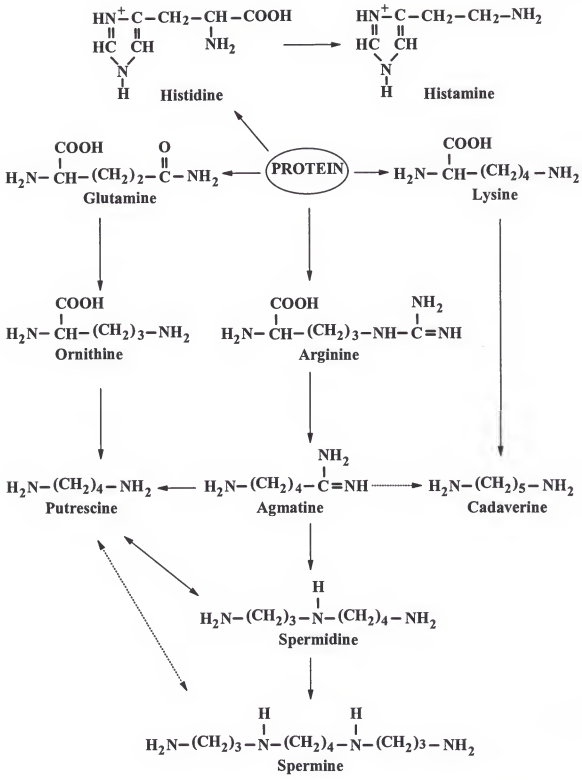


Figure 1. Pathways for the formation of the major biogenic amines - histamine, putrescine, cadaverine, spermidine, and spermine

including fish products, meat products, dairy products, wine, beer, vegetables, fruits, nuts, and chocolate (Brink et al., 1990).

Non-fermented food

In non-fermented foods, the presence of biogenic amines above a certain level is considered indicative of undesirable microbial activity. Therefore, the amine level could be used as an indicator of microbial spoilage. However, the presence of biogenic amines in food does not necessarily correlate with the growth of spoilage organisms because they are not all decarboxylase-positive (Vidal et al., 1990). Levels of histamine, putrescine, and cadaverine usually increase during the spoilage of fish and meat, whereas levels of spermine and spermidine decrease during this process (Brink et al., 1990).

Scombroid fish have most commonly been associated with incidents of histamine intoxication (scombrotoxicosis). The formation of histamine in scombroid and other marine fish containing abundant endogenous histidine has been attributed to microbial action rather than to endogenous histidine decarboxylase activity (Baranowski et al., 1985; Halász et al., 1994). There are two ways in which histidine can be catabolized in fish muscle. The amino acid deamination forms urocanic acid or the histidine decarboxylation forms histamine (Mackie and Fernández, 1977). The deamination activity is the principal reaction under normal physiological conditions; decarboxylation activity can be most important in other circumstances, e.g., bacterial contamination. Several studies of the normal microbial population of marine fish revealed their ability to produce high amounts of histamine at low temperatures (Baranowski et al., 1985; Klausen and Huss, 1987; Halász et al., 1994).

Different biogenic amines (histamine, putrescine, cadaverine, tyramine, spermine, and spermidine) have been detected in fish such as mackerel, sardines, and tuna (Middlebrooks et al., 1988; Ababouch et al., 1991; Wei et al., 1990). Other non-fermented foods such as fruit, juices, vegetables, meat, and milk also contain different biogenic amines in various concentrations (Halász et al., 1994; Romain et al., 1992).

Fermented food

During the preparation of fermented foods, many different kinds of microorganisms can be present. Some organisms are capable of producing biogenic amines. For example, most products in which lactic acid bacteria grow contain considerable amounts of putrescine, cadaverine, histamine, and tyramine (Brink et al., 1990).

After fish, cheese is the next most commonly implicated food item associated with histamine poisoning. The first reported case occurred in 1967 in the Netherlands involving Gouda cheese (Doeglas et al., 1967). In the United States, all of the outbreaks have involved Swiss cheese that contained greater than 100 mg histamine/100 g cheese (Stratton et al., 1991). Two outbreaks occurred in 1976, one of which involved 38 cases (Edwards and Sandine, 1981). Many studies have been undertaken to determine the amine content of cheese products. A variety of amines, such as histamine, tyramine, cadaverine, putrescine, tryptamine, and phenylethylamine, have been found in many types of cheeses (Sumner et al., 1985; Stratton et al., 1991; Besançon et al., 1992; Díaz-Cinco et al., 1992; Moret et al., 1992). Other fermented foods such as sauerkraut, kim chee, miso, soy sauce, dry sausage, anchovies, beer, and wine can also contain high levels of biogenic amines (Brink et al., 1990; Stratton et al., 1991).

Bacterial Species with Amine-decarboxylase Activity

Although amino acid decarboxylases are not widely distributed among bacteria, species of many genera such as *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, and the lactic bacteria *Lactobacillus*, *Pediococcus*, and *Streptococcus* are capable of decarboxylating one or more amino acids (Rice et al., 1976; Brink et al., 1990). *Enterobacteriaceae* are primarily responsible for the decomposition of scombroid fish with the ability to decarboxylate histidine to form histamine (Frank et al., 1985).

It is known that different bacteria vary significantly in either the quantity of decarboxylase they produce and/or the specific activity (turnover number) of those decarboxylases. The type of microflora found on the fish depends on the fish species, geographical location that the fish inhabit (water temperature and microorganisms in the water), season of capture, impact of the vessel that catches and transports the fish, and handling practices (Rogers and Staruszkiewicz, 1997). It is also known that the composition of the fishery product significantly affects the amount of decarboxylase bacteria may release (Rawles et al., 1996).

Organisms like *Morganella* (*Proteus*) *morganii*, certain strains of *Klebsiella pneumoniae*, and a few strains of *Hafnia alvei*, are prolific histamine producers and are important in the microbiology of fish products (Fernández and Mackie, 1979; Stratton et al., 1991). Certain strains of *Lactobacillus* that are also prolific histamine producers may be of importance in fermented fish (Taylor, 1986). *Pleisomonas shigelloides*, a bacterium frequently isolated from fish and aquatic environments, was identified as a new histamine

former in fish by López-Sabater et al. (1994). In addition, bacteria isolated from skipjack tuna (*Euthynnus pelamis*) that are found to produce histamine are *Proteus vulgaris*, *Proteus mirabilis*, *Clostridium perfringens*, *Enterobacter aerogenes*, and *Vibrio alginolyticus* (Yoshinaga and Frank, 1982; Frank et al., 1985). Luminous bacteria can also produce histamine. These organisms are considered halophilic, but both mesophilic and psychrophilic species have been reported (Stratton et al., 1991). Morii et al. (1986) reported that *Photobacterium phosphoreum*, a psychrophile, may be primarily responsible for histamine production in scombroid fish at low temperatures. Among these, the most active and frequent histamine former was *Morganella morganii*, followed in activity by *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and some strains of *Enterobacter cloacae* and *Enterobacter aerogenes*. All these bacteria can produce more than 500 ppm of histamine under experimental conditions (Rawles et al., 1996).

Frank et al. (1985) studied the composition and decarboxylase activity of bacteria isolated from decomposed mahi-mahi. They reported that mesophilic bacteria isolated from fish incubated at 32°C for 24 hr were mainly Gram-negative rods; 89% of these were *Vibrio alginolyticus*. Mesophiles producing strong histamine (>100 mg/100 ml of culture broth) were *Morganella morganii* and *Proteus mirabilis*, while mesophiles producing weak histamine (<10 mg/100 ml of culture broth) were all *Vibro alginolyticus*. Psychrotrophic isolates obtained from fish incubated at 0°C for 14 days were essentially Gram-negative bacteria; 5% of which were *Alteromonas putrefaciens*.

All *Enterobacter aerogenes*, *Pseudomonas fluorescens/putida*, *Pseudomonas putrefaciens*, and *Serratia marcescens* strains produced either putrescine (ornithine

decarboxylase) or cadaverine (lysine decarboxylase). None of the isolated *Morganella morganii* strains, which had the highest capability of producing histamine, could produce cadaverine (Rodriguez-Jerez et al., 1994).

Factors Affecting Amine-decarboxylase Activity

Since amines are formed either by enzymatic activity in the food or decarboxylase active bacteria, inhibition of such activity and prevention of bacterial growth would be very important in controlling the amine content of foods (Silla Santos, 1996). Manufacturing conditions influence the production of biogenic amines. Histamine production in cheese has been related to factors such as availability of substrate, pH, salt concentration, and temperature. The proper storage temperature is probably the most important method of prevention (Stratton et al., 1991).

The pH level is an important factor influencing aminodecarboxylase activity. Amino acid decarboxylase activity was stronger in an acidic environment with the optimum pH between 4.0 and 5.5. Furthermore, bacteria in this environment are more strongly encouraged to produce these enzymes as a part of their defense mechanisms against the acidity (Teodorović et al., 1994).

Storage temperature may affect the content of biogenic amines in food. Low storage temperatures are not sufficient to inhibit the formation of toxic amines (Ababouch et al., 1991; Silva et al., 1998). Baranowski et al. (1985) detected that *Klebsiella pneumoniae* UH-2 produces large amounts of histamine at 10°, 25°, and 37°C. No growth occurred at 2°C, but histamine was found because the resting cells were capable of producing histamine during storage at low temperature. During storage of pork meat at 5°C and -20°C,

putrescine and cadaverine increased, while spermidine and spermine decreased under the same conditions (Halász et al., 1994).

Amine concentrations were unaffected by cooking, with the exception of spermine, which decreased during heat treatment of cooked ground beef at 200 °C for 2 hr (Sayem-El-Daher et al., 1984). Histamine is stable during the cooking process. Once these amines are formed, it is difficult to destroy them. Nevertheless, the original concentration of amines in food can change as a result of storage conditions which should be controlled (Silla Santos, 1996).

Histamine formation was inhibited by salting. During storage of mackerel muscle at 5 °C, very little histamine was formed, regardless of brine concentration. The inhibition effect on histamine was proportional to the increase in brine concentration during storage at 25 °C (Kang and Park, 1984).

Oxygen supply also appears to have a significant effect on the biosynthesis of amines. *Enterobacter cloacae* produces about half the quantity of putrescine in anaerobic compared to aerobic conditions. *Klebsiella pneumoniae* synthesizes significantly less cadaverine but acquires the ability to produce putrescine under anaerobic conditions (Halász et al., 1994).

Function and Physiological Significance

In addition to their biological role as sources of nitrogen and precursors for the synthesis of hormones, alkaloids, nucleic acids, and proteins, amines are also important food aroma components and potential precursors for the formation of carcinogenic N-nitroso compounds (Shahidi et al., 1994). Although polyamines have long been known to be essential for growth, their exact biological role in cell metabolism is still unclear. Due to the

diversity of their roles in cellular metabolism and growth, they are required in rapidly growing tissues in large amounts (Bardócz, 1989). Polyamines are essential for maintaining the high metabolic activity of the normally functioning immunological system of the gut. Spermine and spermidine have also been implicated in the evolution of intestinal tissue (Romain et al., 1992; Bardócz et al., 1993).

Although every cell has some ability to synthesize polyamines, it appears that the body also relies on a continuous supply of polyamines from food, most of which are not retained by the gut tissues but are distributed to different organs in the body. It is likely that there is a body storage pool for polyamines from which they are released in a controlled manner when needed (Bardócz et al., 1993).

Certain classes of amines, the catecholamines, indolamines, and histamine, fulfil important metabolic functions in humans, especially in the nervous system and in control of blood pressure (Halász et al., 1994). Phenylethylamine and tyramine can cause a rise in blood pressure, while histamine reduces blood pressure. Histamine possesses a powerful biological function, serving as a primary mediator of the immediate symptoms noted in allergic responses (Taylor, 1986; Stratton et al., 1991). Putrescine, cadaverine, and agmatine have been identified as potentiators that increase the toxicity of histamine to humans by depressing histamine oxidation (Ibe et al., 1991). The rapid nature of the reaction, in some cases within 5 min, suggests that absorption of at least part of the amine may take place through the oral mucous membrane, bypassing the intestinal aminooxidases (Halász et al., 1994).

Determination of the biogenic amines histamine, tyramine, agmatine, putrescine, cadaverine, spermine, and spermidine is important not only from the viewpoint of their toxicity, but also because they can be used as indicators of freshness or spoilage in foods (Halász et al., 1994). A fishy odor is derived from a variety of components, of which trimethylamine (TMA) is predominant.

Scombroid Poisoning

Scombroid poisoning is a type of food intoxication caused by the consumption of scombroid and scombroid-like marine fish species that have begun to spoil with the growth of particular types of food bacteria that produce toxic quantities of histamine. Fish most commonly involved are members of the Scombridae and Scomberesocidae families (tuna, albacore, bonito, skipjack, mackerel, saury, and wahoo). However, non-scombroid fish, such as mahi-mahi, amberjack, marlin, herring, bluefish, sardines, and anchovies, as well as cheese, have been implicated as causes of scombrotoxism. Scombroid fish poisoning is the most common form of icthyotoxicosis in the world (Morrow et al., 1991; Mack, 1993). In the United States, such poisoning represents one of the major chemical food-borne illnesses reported to the Centers for Disease Control and Prevention (CDC). Between 1973 and 1987, 116 outbreaks (757 cases) of scombroid fish poisoning were reported to CDC. Of these, 109 (94%) involved mahi-mahi, tuna, or bluefish (Bean et al., 1990). Between 1988 and 1992, scombrotoxin accounted for 2.1 to 3.7% of food-borne outbreaks with one case resulting in death (MMWR, 1996).

Clinical Syndrome of Scombrotoxism

Histamine is a powerful biologically active chemical that can evoke many responses within the body. It can directly stimulate the heart, cause extravascular smooth muscle to contract or relax, stimulate both sensory and motor neurons, and control gastric acid secretion (Soll and Wollin, 1977). Thus, it is not surprising that histamine poisoning is often manifested by a wide variety of symptoms. The clinical syndrome of scombrotoxism is usually mild, self-limited, and primarily involves the gastrointestinal and nervous systems and skin. Adverse clinical effects develop in a matter of minutes to a few hours after eating the fish. The initial complaints are headache, diffuse erythema, nausea, vomiting, diarrhea, dizziness, abdominal cramps, burning of the mouth, and dysphagia. Flushing of the face, neck, and upper trunk, bright-red skin rash, sweating, and an oral burning or blistering sensation (peppery tasting) are the most consistent and characteristic symptoms (Dickinson, 1982; Bartholomew et al., 1987). Severe cases may blur vision, cause respiratory stress and swelling of the tongue. Symptoms usually last for approximately 4 to 6 hours and rarely exceed one to two days. Serious complications usually involve the respiratory and cardiac systems. These cases often involve the elderly with underlying cardiac problems or young children with severe asthma (Stratton and Taylor, 1991). Antihistamines such as diphenhydramine, cimetidine, and promethazine have been used successfully in the treatment of patients with scombroid poisoning, shortening the duration of illness (Blakesley, 1983).

Histamine is one of the chemicals released from mast cells during an allergic reaction. It serves as one of the primary mediators of the allergic response (Beavan and Horakova, 1978). Antigens can trigger this response when they come in contact with

antigen-specific IgE molecules on the membrane surface of these cells. Histamine interacts with specific tissue histamine receptors thereby eliciting many of the symptoms associated with allergies (Beavan, 1978). Symptoms for scombroid poisoning can be easily confused with an allergy or other form of food poisoning, and vice versa. Some symptoms for scombroid poisoning are similar to ciguatera, another form of marine fish poisoning. Distinguishing these illnesses can be complicated, particularly around warm water regions.

Constraints to Surveillance

Accurate statistics on the worldwide outbreaks of histamine poisoning do not exist. Many countries do not have adequate systems for reporting foodborne diseases; thus it is impossible to know the prevalence of this illness. In those countries that do report foodborne diseases, cases are probably missed each year for various reasons. Some of those reasons may include the mild nature of the illness, which causes patients not to seek medical attention, or ignorance by physicians who may misdiagnose the illness as a food allergy. Also, histamine poisoning is not a reportable illness even in those countries that keep records (Taylor, 1985).

Formation of Histamine

Histamine is generated in foods from the amino acid histidine via an enzymatic decarboxylation reaction catalyzed by histidine decarboxylase. Certain fish, especially scombroid fish, possess large amounts of free histidine in their muscle tissues (Lukton and Olcott, 1958). This free histidine can serve as a substrate for bacterial histidine decarboxylase. Autolysis or bacterial proteolysis may also be important in that it causes the release of free histidine from tissue proteins (Stratton and Taylor, 1991). Milk protein can

contain large amounts of histidine even though fresh milk has very little free histidine (Sumner et al., 1985). This is important when considering cheese and histamine poisoning. Aged Swiss cheese, for example, may have large amounts of histidine arising from proteolysis (Hinz et al., 1956) that can be converted to histamine by bacterial histidine decarboxylase. Fermented soy products, such as soy sauce and miso, are produced from soybeans which contain considerable amounts of protein that can serve as a substrate for histidine decarboxylase upon proteolysis (Chin and Koehler, 1983a, b).

Histamine formation depends on the free histidine in fish and the histidine decarboxylase activity of bacteria. Histidine decarboxylase is not commonly found within most bacterial groups, however, the enzyme is found in certain *Enterobacteriaceae*, *Clostridium*, and *Lactobacillus* species. Only a few of these species are prolific histamine producers, and these seem to be the ones that cause the problems (Stratton and Taylor, 1991).

Control of Histamine Formation

Several preventive measures can be taken to control histamine formation in foods. The nature of the food product must be taken into consideration in the development of such control measures. With fish, low temperature storage and good hygienic practices are crucial to the control of bacterial histamine formation (Taylor, 1986).

In the fishing industry, low temperature storage after catching is the most important and practical method to control bacterial histamine formation. Histamine-producing bacteria appear to reside in the gills and/or intestines of fish (Frank et al., 1981; Taylor and Speckhard, 1984). Any extended storage of fish at elevated temperatures results in bacterial growth and invasion into the muscle tissues and conversion of histidine to histamine.

Current fishing practices for tuna involve either the immediate icing or storage of the fish in refrigerated seawater before freezing. However, if proper conditions are not met almost immediately, a temperature abuse situation may occur because of the large size of the fish (Stratton and Taylor, 1991).

Histamine-producing bacteria isolated from spoiling fish could most likely be due to postharvest contamination (Taylor and Speckhard, 1984) although they are often considered part of the normal microflora of the fish (Yoshinaga and Frank,1982). However, no proof exists that histamine-producing bacteria are part of the normal microflora of freshly caught fish.

Contamination of fish can occur at any level of processing. They may be contaminated on the fishing boat by human workers or equipment, in the processing plant, or while in the distribution system. Food handlers or consumers may use incorrect practices to store and/or prepare the fish. Proper hygienic practices could control the formation of histamine if postcatch contamination is a major source of histamine-producing bacteria. By monitoring these practices, the resulting improved sanitation would not only decrease the likelihood of histamine poisoning, but also perhaps that of other food-borne illnesses caused by contaminated fish (Stratton and Taylor, 1991).

Histamine Metabolism

Histamine exerts its toxic effects by interacting with receptors on cellular membranes. Two types of histamine receptors (H_1 and H_2) exist in humans and other species (Taylor, 1985). The most common symptoms of histamine poisoning result from the action of histamine on the cardiovascular system, which involves both of these receptors (Beavan and

Horakova, 1978). Histamine-induced contraction of intestinal smooth muscle, mediated by H_1 receptors, may account for abdominal cramps, diarrhea, and vomiting. Pain and itching associated with urticarial lesions may be due to sensory and motor neuron stimulation, which is also mediated by H_1 receptors (Taylor, 1985; Soll and Wollin, 1977; Stratton et al., 1991). Gastric acid secretion is regulated by histamine through H_2 receptors located on the parietal cells. It is unknown whether this action accounts for some of the symptoms observed in cases of histamine poisoning.

Histamine undergoes metabolism primarily by one of two enzymatic pathways (Figure 2). In the first pathway, the ring structure of histamine is methylated by histamine N-methyltransferase (HMT) to form N-methylhistamine which is then oxidized by monoamine oxidase (MAO) to form methyl imidazoleacetic acid. Intravenously or intradermally injected histamine in humans is metabolized primarily by this pathway. In the second pathway, diamine oxidase (DAO) converts histamine into imidazoleacetic acid which can ultimately be conjugated with ribose and excreted. End-products from both enzymatic pathways are excreted in the urine (Taylor, 1986). DAO and HMT are the two enzymes primarily responsible for the metabolism of histamine in the intestinal tract which prevents the absorption of unmetabolized histamine into the circulatory system. The existence of these enzymes (in the absence of potentiators) probably explains why orally administered histamine shows no toxic effects (Hui and Taylor, 1985). However, when activity of these enzymes is suppressed by one or more substances known as potentiators, the detoxification of histamine is inhibited. The presence of potentiators may explain why spoiled fish or aged cheese are more toxic than histamine in aqueous solution (Taylor, 1986).

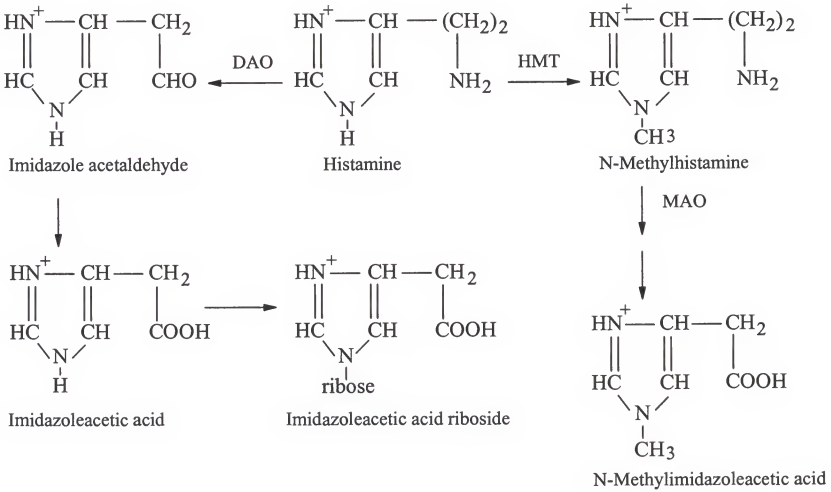


Figure 2. Histamine metabolic pathaways

Histamine Potentiators

Histamine potentiators can be classified as either food-borne putrefactive amines or pharmacologic agents. Some amines, specifically putrescine and cadaverine, inhibit both of the histamine-detoxifying enzymes, DAO and HMT (Hui and Taylor, 1985). Other biogenic amines that may act as potentiators include tyramine, tryptamine, and phenylethylamine. Tyramine can inhibit MAO (Voigt and Eitenmiller, 1978), while tryptamine inhibits DAO (Stratton et al., 1991). Phenylethylamine is a DAO and HMT inhibitor (Hui and Taylor, 1985).

Certain drugs have been implicated as contributing factors in cases of histamine poisoning. Also, certain antihistamines, antimalarials, and other medications can inhibit histamine-metabolizing enzymes (Stratton et al., 1991). Isoniazid, an antituberculosis agent and known inhibitor of DAO, has been implicated in several outbreaks of histamine poisoning (Uragoda and Lodha, 1979; Smith and Durack, 1978).

Analysis of Histamine

Many methods have been developed for the detection of histamine in foods. The earliest methods for histamine analysis were bioassay techniques (Barsoum and Gaddum, 1935), but these methods have been replaced by simpler and more accurate chemical assays. Among the more popular methods for histamine analysis are the fluorometric, chromatographic, and enzymatic procedures.

The AOAC fluorometric procedure is the official method for analyzing histamine in food in the USA (Williams, 1984; Stratton et al., 1991). The method involves extraction of the fish with methanol, separation of histamine from amino acids by passing the extracts

through an ion-exchange column, and reaction with o-phthalaldehyde under controlled conditions followed by fluorometric measurement. This method has been proven to be accurate, sensitive, and reproducible, with a detection limit of approximately 1 mg histamine/100 g fish sample (Gouygou et al., 1992; Staruszkiewicz et al., 1977). The AOAC method employs anion-exchange resin for cleanup, but cation-exchange resins have also been used successfully to remove interfering substances (Lerke and Bell, 1976). Histidine is probably the primary interfering substance in food extracts. The cation-exchange procedure with fluorometric detection of histamine using o-phthalaldehyde is the currently accepted procedure in Germany (Taylor, 1985).

Drawbacks of the AOAC method are that it is rather slow (maximum four or five samples per hour) and requires strict handling. To improve the efficiency and reduce the analysis time required for test samples, numerous analytical techniques, including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), capillary zone electrophoresis (CZE), and enzymatic tests have been developed for the determination of histamine.

TLC methods consist of migration of the food extract on silica gel and then detection of histamine with various spray reagents such as fluorescamine or ninhydrin. These methods are simple but are only semiquantitative. Shalaby (1995) developed a TLC method for analyzing dansyl derivatives of eight biogenic amines: histamine, cadaverine, putrescine, phenylethylamine, tyramine, tryptamine, spermine, and spermidine from fish, cheese, and meat samples. The procedure allowed for the detection of 14 dansyl derived amine samples within 2 hr. Although the method yields only semiquantitative results, it is quite suitable for

routine screening of large numbers of samples. The advantage of using TLC methods is that they do not require any expensive laboratory equipment.

Histamine is usually quantified by HPLC with precolumn or postcolumn derivatization by benzoyl chloride for measuring ultraviolet absorbance, dansyl chloride for measuring visible absorbance, and dansyl chloride or o-phthalaldehyde for fluorescent detection (Walter and Geuns, 1987; Morier-Teissier et al., 1988; Suzuki et al., 1990; Yen and Hsieh, 1991). HPLC procedures are highly specific and usually offer the additional advantages of detecting other amines in the food samples. However, they require expensive laboratory equipment and are time-consuming.

Several GC methods have been developed for detection of histamine and other biogenic amines in foods (Staruszkiewiez and Bond, 1981; Yamamoto et al., 1982). Histamine must be derivatized to a volatile compound in order to be analyzed by GC. A problem with some of the approaches for derivatization of histamine is its incomplete elution from columns and extensive tailing. In spite of their potential application possibilities, GC analysis is still not widely used for histamine in foods.

Another new technique that has been used for histamine analysis is the CZE method (Mopper and Sciacchitano, 1994). This method involves extraction of the fish with 50% methanol and direct injection of the extract onto a fused silica capillary filled with 0.02 M sodium citrate buffer (pH 2.5). The separation of histamine from interfering substances was achieved by applying high voltage between the capillary. The advantages of this method is that it excludes the pre-chromatographic cleanup step which shortens the analytical time. However, the equipment used is still expensive.

To comply with federal regulations, many histamine rapid test kits utilizing enzymatic and colorimetric methods have been developed. These have the advantages of being rapid, do not need expensive instrumentation like HPLC, and are more convenient for use in field application. Most of these tests are based on competitive radioimmunoassays (RIA)(Immunotech, 1996) and enzyme-linked immunosorbent assays (ELISA). Serrar et al. (1995) developed four monoclonal antibodies for histamine analysis using histamine-protein conjugates. All monoclonal antibodies exhibited high affinity for histamine after chemical derivatization and showed no cross-reaction with cadaverine, putrescine, spermine, spermidine, histidine, or serotonine. A competitive inhibition ELISA was developed using these antibodies. The assay has been used to quantify histamine in fish extracts. It showed good correlation with the HPLC method. The advantage of this method was its low detection limit (10 ng/ml). However, the method is complicated and needs to be simplified before applying to a large number of samples.

Hall et al. (1995) reported a solid-phase assay (dipstick test) for histamine in tuna based on the coupling of diamine oxidase to a peroxidase/dye system. This rapid colorimetric dipstick test can be performed within one hour and may be applied to field studies if a portable spectrophotometer is used. The main disadvantage of the test is that putrescine reacted slightly with the dipstick.

The Neogen Alert® for histamine (Neogen® Corporation, Lexington, KY) is another type of method that utilizes the original AOAC principles to perform a rapid histamine test. According to the manufacturer's instructions, a sample extract was prepared using 100% methanol. The extract was then purified using an ion-exchange column followed by reacting

with a diazo dye. The reflectance (intensity of color) was observed and recorded within 5 min using the hand-held AgriMeter II (Agri-Diagnostics Associates, Moorestown, NJ). A positive sample was indicated by color development in the sample port. Color intensity was directly proportional to histamine concentration. Therefore, by comparing the results to the standard solutions and the chart provided by Neogen®, the content of histamine in test samples was determined. Six samples can be analyzed within one hour using this test kit. The procedure is readily portable and inexpensive, however, it lacks the accuracy compared to the AOAC fluorometric method.

Histamine Regulation

The U.S. is one of the few countries that has regulatory limits for histamine. In 1982, the FDA established a defect action level for tuna at 10 mg/100 g (100 ppm) when signs of decomposition are present, 20 mg/100 g (200 ppm) based on histamine alone as an indicator of mishandling, and a hazard action level at 50 mg/100 g as an indicator of a potential health hazard (FDA, 1982). Although the relationship between the level of histamine and the toxicity of a fish sample is not clear, it is conjectured that a histamine content > 200 ppm in fish can be considered toxicologically significant and that a content of > 50 ppm indicates that the fish has been exposed to higher temperatures (Arnold and Brown, 1978; Murray et al., 1982). In France, a maximum permitted concentration of histamine in fresh tuna has been set at 10 mg/100 g (Gouygou et al., 1992).

In 1996, the FDA revised its compliance policy guides on decomposition and histamine to: (1) lower the histamine action level for decomposition to 50 ppm from 100 ppm; (2) extend application of the new action level to raw and frozen tuna and mahi-mahi;

(3) eliminate the provision that findings of less than 200 ppm need to be confirmed by organoleptic testing; (4) allow other species to be considered as decomposed when histamine levels reach 50 to 500 ppm; and (5) consider fish, other than tuna, implicated in histamine poisoning outbreaks as health hazards when histamine levels reach 500 ppm (FDA, 1996).

HACCP Requirements

Due to the impact of new HACCP regulations which became effective on December 18, 1997, there is an increasing demand for new faster methods for determination of histamine content and fish decomposition, which can be accessible to the fisherman or seafood processors for routine analysis. The new HACCP inspection mandate specifically requires monitoring of each critical control point to prevent the potential hazard of food-borne outbreaks associated with elevated histamine levels in fish at receiving and each processing step.

Stated in the Fish and Fishery Products Hazards and Control Guide (FDA, 1998), the HACCP regulations will be affecting importers and processors, both domestic and foreign. The regulation will encompass all fishery products, including the potentially scombrotoxic species. The guidelines require all U.S. bound products to have records of their HACCP plans. If they do not meet the requirements, the entire lot may be subject to loss.

The first step in a HACCP plan is to identify the potential hazard at any processing step which may cause food-borne illness. One of the critical limitations in histamine poisoning is that, once the toxin is formed, it cannot be eliminated by heat (including retorting) or freezing (FDA, 1998). Therefore, preventative measures at the points of

harvest, transportation and processing are the key critical control points for the seafood industry.

Rapid chilling of fish immediately after death is the most important strategy for preventing the formation of histamine. The guideline suggests that the internal temperature of the fish should be brought to 10°C or below within 6 hours of death. Further cooling towards the freezing point is also required to prevent the longer-term, low temperature development of histamine. Once chilled, the fish should be maintained as close as possible to the freezing point (or held frozen) until it is consumed (FDA, 1998).

Detection of scombrotoxin in fish may depend on several factors. Sensory evaluation is generally used to screen suspect fish for spoilage odors that develop when the fish is exposed to time/temperature abuse. However, odors of decomposition may not always be identifiable in fish that have undergone short periods of high temperature spoilage. A complete sensory evaluation should include color and texture as well. Chemical testing is an effective means of detecting the presence of histamine in fish flesh. However, the validity of such testing is dependant upon the design of the sampling plan. Because histamine is generally not uniformly distributed in a decomposed fish, if 50 ppm is found in one section of the fish, there is the possibility that other units of the fish may exceed 500 ppm (FDA, 1998). Therefore, it is important to describe a sampling plan where testing of the entire fish for histamine content is to occur. These records should be included in the HACCP plan (FDA, 1998).

Establishing a recordkeeping system is also required in a HACCP plan for control of histamine formation. These records may include harvest and chill times, time/temperature

indicators during transportation and storage, and sensory examination during all steps until it reaches the end-user.

Assessment of Seafood Freshness

Sensory Evaluation

The freshness of a particular seafood refers to the degree of excellence of that seafood with respect to all sensory variables (appearance, texture, odor, and flavor) normally associated with it. Sensory evaluation of freshness of seafood involves determining the advancement of post-mortem changes in fish and shellfish by using the senses of smell, sight, and touch. The evaluation is in reference to a code containing minimum requirements and, usually, the criteria of quality grades. The most frequently used quality grades are the following: fresh, marketable, and unfit/extra, or A., B., and C. The grade limits do not fully coincide with freshness stages. The first grade usually covers very fresh fish and those with reduced freshness but without any signs of spoilage. Seafoods classified as marketable are those showing the first signs of spoilage. The rejection limit is not well defined due to the difficulties in assessment, particularly with regard to sensory discrimination between fresh and reduced fresh fish. To make the assessment more definite, five or ten point scales are often used. The scale allows one to evaluate some of the most important characters, such as raw odor or flavor, to arrive at the final grade. Descriptive analysis is often used to select the various important characters. Another type of grading involves assessing defects and their intensity. Each quality class corresponds to a certain score or allowable defects (Sikorski et al., 1990).

Fish freshness is traditionally judged by sensory methods. A proper sensory analysis requires a high degree of standardization, training, and control (Farn and Sims, 1986). Trained and experienced judges must be used to increase objectivity and reduce error. Attempts have been made to combine sensory assessment with objective microbiological, chemical, or physical tests.

Microbiological Tests

It is required that a freshness test be fast, reliable, consistent with sensory assessment, and can be applied to all seafoods. This is difficult to achieve in view of the variety of seafood species and biochemical changes.

Microbiological tests based on the determination of the total number of bacteria are of little use. The total bacteria count for the fish just after catch depends mainly on environmental pollution and temperature of the environment, on the method of catching, and on the conditions of handling aboard the vessel. The water in the open sea contains very small numbers of bacteria, i.e., a few colony-forming units (cfu) per 1 ml, while the coastal regions and the sediments may be heavily polluted, up to about 10^6 cfu per ml. Handling and storage of the catch on ice brings about a change in the number, distribution, and composition of microflora on seafoods. The growth of bacteria on fish can be generally represented by a sigmoidal pattern. The initial lag period lasts usually until the resolution of rigor mortis. The freshness of fish stored on ice may decrease while the total surface bacteria remain the same (Sikorski et al., 1990).

Generally, the composition of microflora is also determined in the freshness test, e.g., the number of psychrotropic Gram-negative organisms, hydrogen sulfide producers, and

indicator bacteria. The skin microflora of fresh fish taken from cold waters is dominated by psychrotropic Gram-negative bacteria (Sikorski et al., 1990).

Chemical Methods

Chemical indices, indirectly related to bacterial activity, are often used in assessing freshness. Numerous chemical indicators such as ammonia, trimethylamine (TMA), total volatile bases (TVB), volatile acids, volatile reducing substances (VRSs), pH, buffering capacity, sulfides, nucleotide breakdown products, amino acids, hypoxanthine, indole, skatole, K value, biogenic amine, and others, have been proposed. In general, most of the chemical tests appear to have variable success, with limitations being placed on them by innate biological differences from species to species. No single indicator, however, is sufficient to qualify a seafood as fresh. Most often at least two tests are used, one to determine the loss of freshness, and the other to detect bacterial spoilage (Rosier and Peteghem, 1988; Sikorski et al., 1990).

Two common classes of organic bases found in fish and shellfish are trimethylamine oxide (TMAO) and betaines. TMAO and its breakdown products are among the most studied compounds relative to the composition and decomposition of seafoods. TMA could be formed from TMAO as a result of bacterial enzyme activity. Changes in TMA content in fish with time of storage correlated with bacterial count and sensory score, particularly with raw odor. Therefore, a high level of TMA would be an indicator of the degree of bacterial spoilage. Dimethylamine (DMA) is also produced from TMAO, but by the enzyme activity of fish flesh. Only when bacterial growth is halted (e.g. by freezing) does the DMA content increase (Sotelo et al., 1995). It is suggested that quality control of frozen fish

should be based on both TMA and DMA; TMA as a prefrozen quality index and DMA as an index of frozen storage deterioration.

Adenosine triphosphate (ATP) breakdown, from ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx), can reflect the first changes in fish before bacterial growth. IMP is formed through autolysis. The disappearance of IMP may be used to measure changes in freshness quality that occur during the early stages of chilled storage (Hattula et al., 1993). However, the formation of Hx from HxR is an indication of both initial autolytic changes and later changes caused by bacterial spoilage. It can also be used as a fish hygienic quality indicator (Veciana-Nogués et al., 1997). Both the disappearance of IMP and the formation of Hx have been observed to be quite variable between individuals within a species as well as between species (Price et al., 1991). In order to overcome these variabilities, the K-value, i.e., percent ratio of HxR and Hx to the sum of ATP and all the products of ATP degradation, was proposed by Satio et al. (1959) as an index for estimating the freshness of fish. It is widely used in Japan where many fish species are caught and commercially used.

$$K\% = \frac{HxR+Hx}{HxR+Hx+ATP+ADP+AMP+IMP} \times 100$$

Biogenic amines can also be useful in estimating freshness or degree of spoilage of fish. These compounds are found at very low levels in fresh fish. Their formation is associated with bacterial spoilage (Fernández-Salguero and Mackie, 1987b). Furthermore,

biogenic amines can be useful as indicators of poor-quality in raw material for preserved fish products because they are thermally stable compounds (Sims et al., 1992).

Physical Tests

Physical methods such as electric resistance, toughness of meat, turbidity of crystalline lens, color, and water-holding capacity have been proposed for determining freshness of fish (Ohashi et al., 1991). Among the physical methods for freshness assessment, texture measurement is applied most often. Texture features can be determined by the puncture force or by measuring the rheological changes brought about by the applied force. The main problem associated with instrumental measurements in freshness assessment is the difficulty of finding the sensory equivalence to the measured physical parameters (Sikorski et al., 1990).

Various electronic devices, such as the Torry fish freshness meter and the Intelectron fish tester, have been developed for rapid estimation of fish freshness on board vessel and in the fish market. These devices are simple to operate and some of them can be applied even to fish already packaged (Burt et al., 1976).

Electronic Nose Technology

Odor/aroma is an important quality attribute of food. Smell is one of the most potent human senses. However, smell has proven to be difficult to quantify. Aromas and odors consist of hundreds of different volatile compounds and the human response to odors changes with age, health, habits, and background. Advances in organic chemistry, electronics, and computing in recent years have combined to make possible the development

of new digital aroma technology (electronic nose) which mimics the human nose (Russell, 1995).

Principles of Olfaction

To understand how an electronic nose operates, it is necessary to have a basic understanding of the principles behind the human olfactory system. The sensation of smell is dependent upon the interaction of odorant molecules with a group of specialized nerve cells, the olfactory receptors, which are situated just above the bridge of the nose out of the main air passage. The hydrophobicity of a molecule is important since the first step in the process of olfactory recognition is the dissolution of the molecule in an aqueous mucous layer covering the olfactory receptor cells. Each olfactory cell has a number of cilia, effectively increasing the surface area, which contain different guanine nucleotide-binding proteins (Breer, 1994; Clapham, 1996).

The significance of the sense of smell is illustrated by the sheer weight of genetic information used to control it. About 1,000 genes encode 1,000 different odor receptors. The odor receptor genes account for approximately 1% of all human genes. The olfactory receptor forms a pocket-like structure into which a wide variety of odor molecules may be specifically bound (Breer, 1994).

Many theories, such as the stereochemcial theory of Amoore (1970) and the vibration theory of Wright (1982), have been proposed to explain the process of olfactory recognition. The exact method of interaction between an odor molecule and a receptor site has not been proved conclusively, but it is known that interaction results in excitation of the receptor cell. This produces a cascade of reactions which actively control ion channels within the cell

membrane and results in an electrical signal that is passed along the axon to the olfactory bulb. Two transduction pathways have been identified using separate second messengers, cAMP and inositol triphosphate (IP_3), which are thought to act in opposition to each other (Vogt et al., 1990; Breer, 1994).

The human olfactory system consists of about 1,000 distinct types of binding proteins and about 100 million olfactory cells. Humans can detect at least 10,000 odors and identify (describe) approximately 50 odors (Bartlett et al., 1997).

The olfactory bulb is composed of three main layers: glomeruli, mitral cells, and granular layer. Each receptor type is linked to a specific group of glomeruli. Thus, stimulation of a combination of olfactory receptors results in the formation of a two-dimensional topographical map representative of a particular odor. The electrical signals produced are further processed by the mitral cells and finally sent via the granular layer to the brain. The overall function of this stage is to reduce the noise associated with the signal and amplify it, effectively increasing both the sensitivity and the selectivity of the system (Bartlett et al., 1997).

Sensory and Instrumental Techniques

Odorant molecules are generally small (molecular weight 20-300 Daltons), polar, and can be detected by humans at levels below 1 ppb. Most natural aromas are complex mixtures of chemicals. Odor perception depends upon mutual interactions (additive, synergistic, antagonistic, or compensative) of these compounds (Bartlett et al., 1997). Sensory analysis is the most widely used technique for odor analysis. Despite having high performance at low concentrations, the human nose cannot be used easily for odor monitoring in the laboratory

and for daily routine analyses. Current research seems to indicate that no two humans really perceive the same aroma quite the same, nor can they communicate a common descriptor easily due to an array of cultural, presuppositive, and associative memory effects. Therefore, traditional sensory evaluation is usually highly subjective, prone to error, and is difficult to relate to other analyses (Strassburger, 1996).

Physicochemical techniques such as GC, GC/O (gas chromatography linked to olfactometry), gas chromatography/mass spectroscopy (GC-MS), and others have been used in industry for more objective analysis. These analytical techniques can objectively discriminate odors and quantitatively measure individual compounds. However, the sample must be separated into its individual components before analysis. All odors are volatiles, but not all volatiles are odors. GC cannot conclude if volatiles are flavor-active. Therefore, all volatile molecules, responsible for the odor or not, are identified and quantified by these techniques (Fernandez et al., 1994). Furthermore, some flavor-active components are present in concentrations below detection limits of GC-MS systems. For industry, the main inconvenience of these methods is their cost and difficulty to correlate to perceived odors.

Considerable interest has recently arisen in the use of an array of gas sensors coupled with a pattern recognition technique to identify vapors and odors based on human response. The sensors, also called an electronic nose, are less sensitive than the human nose, but they are able to give a good response to characterize a global mixture of odors (Fernandez et al., 1994). The electronic nose could provide the bridge between chemical and sensory analysis.

History of the Electronic Nose

In 1964, Wilkens and Hatman developed the first complex system based on redox reactions of odors at an electrode. A sensor array used for discrimination between odors was first demonstrated by Persaud and Dodd in1982, at Warwick University, United Kingdom (Gardner et al., 1990). The first conference dedicated to the electronic nose was held at Iceland in 1990. Subsequent improvements in both sensors and data analysis methods have led to the development of several commercial electronic noses. Electronic nose technology literally exploded in the United States in late 1994. Reports appeared in Fortune and The Wall Street Journal, as well as in the food and flavor trade journals, applauding how the technology mimics the human nose (Strassburger, 1996).

There are three commercial systems, Alpha M.O.S. (France), AromScan (United Kingdom), and Neotronics (United Kingdom) currently manufacturing and selling noses for the industry. There are various other gas sensing systems from other venders; however, they are not typically packaged with the application software and automation features to be employed in aroma analysis (Strassburger, 1996).

Comparison of Human Nose and Electronic Nose

The human nose uses a complex system of interconnected receptors and neurons, which conduct signals to the brain for interpretation. The signals from these nerves travel directly to the limbic system in the brain. When an aroma is sensed, the molecules from the vapor interact with numerous receptors, causing them to send a signal to the brain. The pattern of signals is recognized and interpreted by the brain based on prior training.

An electronic nose works in a similar fashion. An array of chemical sensors is used to mimic a group of protein receptors in the human nose and produces a time-dependent electrical signal in response to an odor. The signals from these sensors are connected via electronics to a microprocessor or computer. Any noise and sensor drift may be reduced using signal preprocessing techniques. The electronics replace the neurons in the olfactory limbic system and the computer or microprocessor acts as the brain. The signals that are sent to the computer can be interpreted using special pattern recognition software (Bartlett et al., 1997). Figure 3 illustrates the basic components of the human olfactory system and relates these to the corresponding components of an electronic nose.

Type of Sensors

The electronic nose system relies on an array of chemical sensors which collect chemical data above the headspace of a sample. When appropriate chemical sensors are exposed to a sample, each sensor exhibits a characteristic response dependent upon the chemical interactions between the sample and the sensors. The sensor array used in the electronic nose system will ideally provide a response to all compounds in the sample headspace which contribute to the sample odor. The number of sensors in the array needs to be small for ease in data interpretation and processing. Therefore, it is necessary for each of the sensors in the array to be somewhat non-specific, providing responses to more than a single species (Neotronics Scientific, 1996). The main types of sensors used in the commercial electronic nose are conducting polymers, semiconductor metal oxides, and quartz resonator sensors (surface acoustic waves sensor and quartz crystal microbalance).

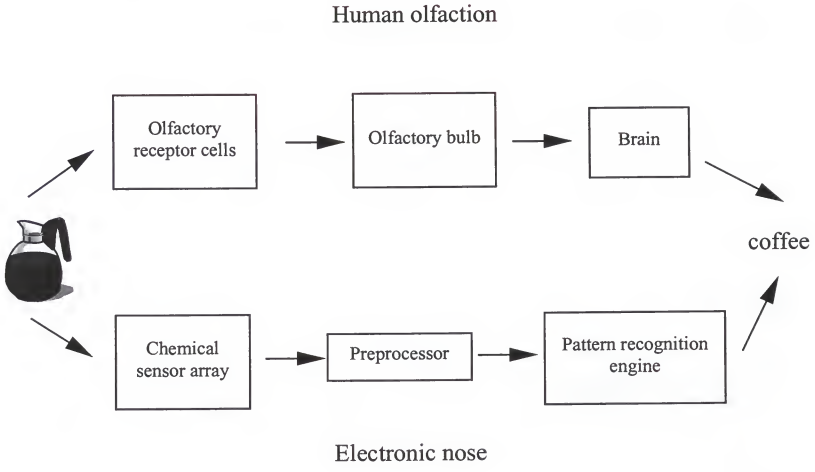


Figure 3. A comparison of the basic elements of the human olfactory system with an electronic nose

Other types of sensors have been applied including: optical, fibre optic, catalytic metal, and electrochemical sensors (Stetter et al., 1986; Dickinson et al., 1996).

Conducting polymer sensors

Many polymers, such as polypyrrole, polyaniline, and polythiophene, are electrically conductive under the right conditions. These polymers may be deposited between two electrodes and used as gas sensors. A change in the conductive properties of the polymer results from exposing the sensor to a gas species. This change may be monitored by passing a current between the two electrodes and observing a change in the voltage across the conductive polymer. The conductivities of the polymers change rapidly and reversibly in the presence of odors. The absorbed odor molecules cause a swelling of the polymers and interfere with charge transfer within the polymer (Neotronics Scientific, 1996; Bartlett et al., 1997).

There are two ways of constructing conducting polymer sensors. The first involves placing a base layer of polymer (generally polypyrrole) between the two electrodes via either an electrochemical or a lithographic technique. A layer of a functionalized polypyrrole is then placed on top of the base layer using a lithographic process. Selectivity of a given sensor is controlled by using different chemical functional groups on the top layer of polymer. The second involves single step electrochemical growth of an entire sensor. Two electrodes are immersed into a solution of a prepolymer (often pyrrole), a solvent, and an organic or inorganic salt. The conducting polymer is electrochemically grown out of solution and fills the gap between the two sensor electrodes. The selectivity of the polymer sensor is achieved by altering the nature of the anions (Neotronics Scientific, 1996).

Regardless of their means of production, conducting polymer sensors age with time to some degree. Calibration algorithms must be used in order to compensate for drift which occurs over long periods of time (Neotronics Scientific, 1996). Conducting polymer sensors are the most popular sensors used in the electronic nose because of their high sensitivity (0.1-100 ppm) to a broad range of substances, ease of signal detection and the possibility of tailoring their chemical structure and properties according to the particular odor-sensing application. These sensors can operate at room temperature and can be easily reproduced by electrochemical deposition. The major commercially available electronic nose based on this technology is AromaScan which consists of 32 conducting polymer sensors (Bartlett et al., 1997).

Semiconductor metal oxide sensors

Metal oxide gas sensors are based upon ceramic sensing elements such as tin oxide or zinc oxide. The oxide materials contain chemisorbed oxygen species with which interaction of odor molecules alters the conductivity of the oxide. These sensors operate at elevated temperatures (350-600°C) to avoid interference from water and to aid in rapid response and recovery times. This results in a high power consumption (500 mW). They are quite sensitive to combustible materials, such as alcohols, but are less sensitive at detecting sulfur- or nitrogen- based odors (Bartlett et al., 1997). Metal oxide sensors are often subject to poisoning, especially by high molecular weight species. In addition, metal oxide sensors are generally less selective and specific than many other sensors (Neotronics Scientific, 1996). The main commercially available electronic nose based on this technology is the Intelligent Electronic Nose (Alpha M.O.S. America, Inc., Belle Mead, NJ). It consists of 6,

12, or 18 metal oxide sensors. Conducting polymer and surface acoustic wave sensors can also be used in this instrument.

Quartz resonator sensors

A quartz resonator sensor consists of a piezoelectric quartz crystal oscillator coated with a sensing membrane, such as acetyl cellulose or lecithin. Adsorption of odor molecules on the membrane leads to changes in the resonant frequency of the device due to a change in mass. Surface acoustic waves sensor (SAW) and quartz crystal microbalance (QCM) are two main types of quartz resonator sensors used. The coatings used for QCM and SAW are often similar materials. It is by the selection of different coating membranes that specificity in response is obtained. QCM sensors have been used in electronic nose arrays by coating them with stationary phases used in gas chromatography. SAW generally operate at much higher frequencies (250 MHz) than do QCM (10 MHz). The greatest drawback for the application of these technologies is their low reproducibility in sensor production (Neotronics Scientific, 1996; Bartlett et al., 1997).

Electronic Nose Data Analysis

Numerous pattern recognition techniques have been used for electronic nose data analysis. They are classified as model-based and model-free or supervised and unsupervised methods. The term model-based describes a situation in which the correlation between measured sensor values and concentrations of gases is functional and may be described by a calibration function with a precisely defined set of parameters. In contrast, the model-free approach only utilizes an implicit representation of the correlation without a definition of parameters with physical meaning (Göpel, 1995).

The further classification of the different methods by supervised and unsupervised approaches refers to the specific way in which the calibration is performed. Supervised algorithms require the determination of sensor signals, as well as concentration values, of a calibration data set during the calibration. The calibration information is obtained by a comparison of sensor signals and concentration values (e.g., by linear or non-linear regression, Kohonen nets, etc.). Therefore, supervised methods may be used for both qualitative and quantitative analysis of mixtures. In contrast, unsupervised methods require only sensor signals for calibration. During calibration, sensor signals are correlated with different classes by applying various criteria to define similarities or differences. In this case, qualitatively operating algorithms which aim at a classification of the sensor signals are chosen (Göpel, 1995).

Principal components analysis is classified as a model-based supervised approach for qualitative analysis. Multivariate linear regression (MLR), principal components regression (PCR), partial least squares regression (PLS), as well as discriminant analysis, are classified as model-based supervised approaches for quantitative analysis. The main representatives for model-free unsupervised approaches for qualitative analysis are the cluster analysis (CA) and self organizing feature map (SOM). The artificial neural networks which can handle non-linear data and that are more tolerant to sensor drift and noise are a representative of model-free supervised approaches for quantitative analysis (Göpel, 1995; Neotronics Scientific, 1996).

Principal components analysis

The purpose of principal components analysis (PCA) is to take a set of variables (the individual sensor responses) and produce combinations of variables (principal components) which are not correlated. These principal components can also be termed as functions. These functions account for variability in the data set. Each function accounts for some measure of difference between samples in the data set. These functions may be plotted against each other. The relationships between samples can be observed graphically on a scatterplot. For example, in a PCA plot, the first principal component (function) might represent differences between sample odors while the second principal component describe differences between replicates of the same sample (Neotronics Scientific, 1996).

Discriminant analysis

In discriminant analysis, the X and Y functions are developed. They account for differences between different samples rather than replicate to replicate differences (system or method parameters). In order to do this, the computer needs a set of training data representative of each of the sample odors. For the training data, the computer needs to be advised of which replicates belong to which classification. The coefficients in the X and Y equations can then be developed to account for differences between sample classifications rather than for other measurements which do not correlate with odor. If the analysis is successful, a plot can produce and display differences between samples. The training model developed can then be used for processing the data obtained from unknown or test samples. This is displayed graphically on the same plot as the training data. The unknown sample may

fall into or near a cluster of training data, indicating its similarity or classification into that sample type (Neotronics Scientific, 1996).

Artificial neural network

An artificial neural network (ANN) is an information processing paradigm that was inspired by biological nervous systems, such as the brain, that process information. The key element of this paradigm is the novel structure of the information processing system. It is composed of a large number of highly interconnected processing elements (neurons) working in unison to solve specific problems. An ANN is configured for an application such as identifying chemical vapors through a learning process. Learning in biological systems involves adjustments to the synaptic connections that exist between the neurons. This is true of ANN as well. For the electronic nose, the ANN learns to identify the various chemicals or odors by example (Sablani et al., 1995; Neotronics Scientific, 1996).

ANNs have been applied to an increasing number of real-world problems of considerable complexity. Their most important advantage is in solving problems that are too complex for conventional technologies - problems that do not have an algorithmic solution or for which an algorithmic solution is too complex to be found. In general, because of their abstraction from the biological brain, ANNs are well suited to problems that people are good at solving but computers are not. These problems include pattern recognition and forecasting. However, unlike the human capability in pattern recognition, the ANN's capability is not affected by factors such as fatigue, working conditions, emotional state, and compensation (Huang et al., 1993).

Industry Application

Currently, the biggest market for electronic noses is the food industry. In some instances, electronic noses can be used to augment or replace panels of human experts. In other cases, electronic noses can be used to reduce the amount of analytical chemistry that is performed in food production, especially when qualitative results will do. Potential applications of electronic noses in the food industry are numerous. Existing applications include: fish and meat quality/control, fermentation control, measuring freshness/storage of fruits and vegetables, checking mayonnaise for rancidity, automated flavor control, monitoring cheese ripening, verifying if orange juice is natural, beverage container inspection, product packaging interaction, microwave oven cooking control, freshness analysis of snack products and determination of shelf life, grading of whiskey, beer, coffee blends or beans, classification of ground black pepper, and adulteration of parsley (Gardner, 1994; Large, 1995).

Other areas where electronic noses have been applied include: chemicals, cosmetics and toiletries, pharmaceuticals, packaging, environment and waste treatment, and medical diagnostics. Environmental applications of electronic noses include: identification of toxic wastes, analysis of fuel mixtures, detection of oil leaks, identification of household odors, monitoring air quality, monitoring factory emission, and testing ground water for odors (Hobbs et al., 1995).

Because the sense of smell is an important sense to the physician, an electronic nose has applicability as a diagnostic tool. An electronic nose can examine odors from the body (e.g., breath, wounds, body fluids, etc.) and identify possible problems. Odors in the breath

can be indicative of gastrointestinal problems, sinus problems, infections, diabetes, and liver problems. Infected wounds and tissues emit distinctive odors that can be detected by an electronic nose. Odors coming from body fluids, such as blood and urine, can indicate liver and bladder problems. The electronic nose has been used to track glucose levels in diabetics, determine ion levels in body fluids, and detect pathological conditions such as tuberculosis (Wang et al., 1997; Schiffman et al., 1997).

CHAPTER 3
MATERIALS AND METHODS

Fish Preparation and Storage

Storage of Salmon at -20°, 4°, and 10°C, or 1.7°, 7.2°, and 12.8°C

Fresh Atlantic salmon (*Salmo salar*, whole fish but eviscerated) were purchased from a local seafood store in Gainesville, Florida. Following washing under running tap water, the salmon was filleted to prepare skinless samples (15 x 12 cm) of about 280 g each. After each fillet sample was placed and labeled in separate sterile Whirl-Pak® bags (Fisher Scientific, Orlando, FL), they were divided randomly into three groups and stored at -20°, 4°, and 10°C for 3, 5, 7, 9, and 14 days. Storage at -20°C was achieved by placing the salmon samples in a styrofoam box and storing in a -20°C walk-in cold room. The 4°C storage was done in a seafood display chamber in which the salmon fillets were covered with crushed ice. The 10°C storage was achieved using a temperature-controlled (10°C) refrigerator.

At each sampling interval, eight pieces of salmon fillets were removed from each temperature group. Four pieces were used to determine bacterial counts, histamine level, and sensory quality using a 10-person taste panel. The remaining four pieces were analyzed using an AromaScan. Six pieces of salmon fillets were used as day 0 control to determine background bacterial loads and sensory quality both by the taste panel and AromaScan. The experiment was replicated a second time.

In another salmon storage study, Atlantic salmon (*Salmo salar*) aquacultured at Puerto Montt, Chile, was harvested and prepared on the same day as fresh, skin-on fillets through an arrangement between Dr. W. S. Otwell and the Chilean Salmon Association. Chile was chosen for obtaining salmon samples due to their quality reputation through controlled production and harvest and their evident growth in long-distance fresh exports about the world. Salmon fillet imported to the U.S. in 1995 were valued at $51 million, compared with less than $1 million in 1994 (USDA Aquaculture Outlook Report, 3/7/96). Chile contributed over 85 percent to the imported fillets market.

Salmon were packaged and shipped by traditional methods and routes by the participating firm in Chile. Six fillets (each 7 to 8 pounds) were individually wrapped in plastic and packaged in layers between crushed ice in a polystyrene box (inside diameters: L80 x W20 x D16.5 cm with 2.0 cm wall thickness for the box and lid). The temperature of the packaged fillets was monitored using VITSAB's visual time-temperature integrator (TTI's, Malmo, Sweden). The lid was sealed tight with tape. Time from harvest to sealed boxes was less than 24 hr. The sealed boxes were transported by truck to Santiago, Chile, for air transport to Miami, Florida, where they were retrieved by Dr. Otwell for ground transport to Gainesville, Florida.

On arrival in the Food Science and Human Nutrition Department, University of Florida, the boxes were opened and the salmon was examined for product quality. The internal box temperature was measured. All salmon fillets within each box were examined for sensory attributes. The internal meat temperature was measured by a hand-held probe thermometer.

The fish fillets were cut into 5-inch-long fillet sections and placed directly into plastic tubs (six fillets per tub). Three groups of fresh salmon fillets (6 pieces per group) were then stored at 1.7°, 7.2°, and 12.8°C (35°, 45°, and 55°F) continuously. One group of fillets was stored at 1.7°C for 1 day, then shifted to 12.8°C for 24 hr before returning to 1.7°C (Temp. Ramp I). A fifth group was stored at 1.7°C for 2 days, then shifted to 12.8°C for 24 hr before returning to 1.7°C (Temp. Ramp II). The last two groups were used to simulate temperature abuse conditions. Sensory, aroma, and bacteriological evaluations of samples were carried out at 0, 1, 3, 5, and 7 days for determination of the time-temperature storage conditions on the quality of these aquatic food products.

Storage of Mahi-mahi at 1.7°, 7.2°, and 12.8°C

Fresh mahi-mahi (dolphin fish, *Coryphaena hippurus*) was obtained from commercial liaisons in the Gulf of Mexico through the help of Dr. W. S. Otwell. Three groups of fresh mahi-mahi fillets (6 pieces per group) were processed and stored at constant temperatures of 1.7°, 7.2°, and 12.8°C. Another group of fillets was stored at 1.7°C for 1 day, then shifted to 12.8°C for 24 hr before returning to 1.7°C (Temp. Ramp I). A fifth group was stored at 1.7°C for 2 days, then shifted to 12.8°C for 24 hr before returning to 1.7°C (Temp. Ramp II). Samples were examined at 0, 1, 3, and 5 days for determination of the effect of various time-temperature storage conditions on the quality of these aquatic food products by sensory, aroma, bacteriological, and chemical evaluations.

Storage of Yellowfin Tuna at 0°, 4°, 10°, and 22°C

Fresh yellowfin tuna (*Thunnus albacores*) loins of 15-20 kg each were purchased from a local seafood store in Gainesville, Florida, and transported in ice to the Food Science

and Human Nutrition Department, University of Florida. The outer layers of the tuna loin were then carefully removed with a sterile knife. The loins were cut to prepare 120 pieces of tuna steak (12 x 10 x 2 cm) of about 250 g each. After each steak was placed in separate sterile, labeled Zipper bags (26 cm x 28 cm, Tenneco Packaging, Pittsford, NY), the samples were divided randomly into four groups and stored at 0°, 4°, 10°, and 22°C for 1, 3, 5, and 9 days.

At each sampling interval, five pieces of tuna steaks were removed from each temperature group to determine bacterial counts, biogenic amine level, and sensory quality using a 10-person taste panel and an AromaScan. Five pieces of tuna steaks were used as day 0 control to determine background bacterial loads, biogenic amine content, and sensory quality both by the taste panel and AromaScan.

Microbiological Analysis

Total Microbial Counts

For total microbial counts, about 20 g portions of fish fillet were cut from test samples at each sampling period and homogenized at high speed for 2 min in a sterile blender with nine volumes (1:9, w/v) of sterile Butterfield's buffer. The homogenates were serially diluted with sterile Butterfield's buffer. Then, 0.1 ml aliquots of the diluents were surface plated on quadruplicate aerobic plate count (APC) agar (Difco, Detroit, MI) plates containing 1.5% NaCl. Pour plate method was also used for some homogenates as necessary. Bacterial colonies were counted after the plates were incubated at room temperature for 48 hr.

Isolation and Identification of Histamine-Producing Bacteria from Salmon and Tuna Fillets

The same homogenates used for total microbial counts for salmon and tuna samples were also plated on Niven's differential medium (Niven et al., 1981). This media was previously shown to accurately detect 95.8% of histamine-producing bacteria on temperature abused tuna (Chen et al., 1989). Four plates were used for each dilution. Following incubation at 37°C for 24-48 hr, two colonies showing deep-blue to purple color were picked from each differential agar plate. These were streaked onto trypticase soy agar plates (TSA, Becton Dickinson, Cockeysville, MD) containing 1.5% NaCl for maintenance and for examination of colony size, morphology, and color. Colonies with typical morphology were chosen, inoculated into both TSA and Niven's medium, and incubated at 37°C for 24 - 48 hr. After being reconfirmed by Niven's medium as a positive specie, isolated single colonies from TSA plates were selected and used for Gram staining and then tested for biochemical characteristics using the API 20E strips (bioMérieux, Hazelwood, MO). Following incubation at 37°C for 24 hr, the seven-digit numerical profile result of each culture was compared with the directory of the Analytical Profile Index (Analytab Products). The cultures were also tested with API OF (oxidation-fermentation glucose) and M (motility) media for further identification. MacConkey agar plates were also used when necessary for differentiating between colonies of similar appearance. It provided a degree of selectivity for Gram negative organisms.

Histamine Analysis

In order to determine if histamine concentrations correlate with fish spoilage, samples were collected on various days for histamine analysis. Salmon fillets were collected on day 7, 9, and 14 of storage at -20°, 4°, and 10°C. Mahi-mahi samples were collected on day 0, 1, 3, and 5 following storage at 1.7°, 7.2°, and 12.8°C. Tuna samples were collected on day 0, 1, 3, 5, and 9 following storage at 0°, 4°, 10°, and 22°C.

Preparation of Histamine Standard Solutions

Histamine stock solution (1000 ppm) was prepared by dissolving 16.6 mg histamine dihydrochloride (Sigma, St. Louis, MO) into 10 ml volumetric flask with 50% methanol. Standard histamine solutions (0.5, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 ppm) for CE analysis were prepared by diluting the stock solution with 50% methanol.

Extraction of Histamine from Salmon and Mahi-mahi Samples

The method of Mopper and Sciacchitano (1994) with slight modification was followed. On the day of testing, duplicate 10-g portions were cut from every fillet sample of each group and blended separately in half-pint Mason jars with 25 ml of 50% methanol at low speed for 30 sec and then at high speed for 1 min. For fortified samples, aliquots of histamine standard solutions were pipetted onto fresh fish samples to make the final histamine concentration of 25, 50, 125, 250, and 500 ppm before proceeding to extraction. The homogenate was carefully transferred from the jar to a 50 ml volumetric flask with two rinsings using small portions of 50% methanol. After the flask was placed in a 60°C water bath for 15 min and the flask cooled to ambient temperature, the volume was adjusted to 50 ml using 50% methanol. The sample was filtered through Whatman #1 filter paper (Fisher

Scientific, Orlando, FL) and then a 0.8 μm filter. The filtrate was then injected into the CE. Control blanks and fortified samples were extracted similarly.

Capillary Electrophoresis (CE) Determination of Histamine

CE was performed on a BioFocus 2000 Capillary Electrophoresis System (Bio-Rad, Hercules, CA) using a coated capillary cartridge of 24 cm x 25 μm I.D. The cartridge temperature was maintained at 35°C. Before injection, the capillary cartridge was rinsed with 0.1 N NaOH for 1 min, then with water for 2 min, and finally running buffer (0.02 M, pH 2.5 sodium citrate buffer) for 3 min. Sample solutions were injected (8.4 nl aliquot) into the capillary by low pressure injection at 20 psi*sec. A constant-voltage at 10 kV was applied and detection of histamine was performed by monitoring the absorbance at 210 nm. Data were stored in a Pentium PC and processed using an integration BioFocus program (Bio-Rad).

Histamine standards were analyzed together with test fish samples (blank, fortified, and test samples). During analysis, a standard solution was also injected intermittently along with test samples to check chromatographic consistency. Each sample was injected twice. Peak areas of histamine standard solutions were used to prepare a standard curve. From the standard curve, histamine concentrations in test samples were calculated. At the end of each experiment, the CE was run with a shut down cycle.

Histamine Analysis of Tuna Using a Fluorometric Method

A modified AOAC fluorometric method for histamine analysis (AOAC Official Method 977.13, 1990) using 75% methanol as the extracting solvent was used for sample extraction (Rogers and Staruszkiewicz, 1997). To each duplicate minced tuna sample (10

g) in a half pint Mason jar, 60 ml of 75% methanol was added, blended at low speed for 30 sec and at high speed for 1 min. The homogenate was transferred to a 100 ml volumetric flask. The Mason jar and blade were rinsed with 10 ml of 75% methanol solution twice and the solutions were pooled into the volumetric flask. The flask was then left in a 60°C water bath for 15 min. After the solution was cooled to room temperature, the volume was adjusted to 100 ml with 75% methanol. The solution was mixed and filtered through Whatman #1 filter paper and then a 0.2 μm filter. Methanol extracts were kept in the refrigerator until further analysis (Figure 4).

The extracts were subjected to chromatography using a Dowex 1-X8 cartridge column. After washing the column with 10 ml of H_2O, 4-5 ml of H_2O were passed through the column and the eluate discarded. One ml of extract was pipetted onto the column and 4-5 ml of H_2O were added. Column flow was immediately initiated into a 50 ml volumetric flask containing 5 ml of 1.0 N HCl. When the liquid level was about 2 mm above the resin, 5 ml of H_2O were added and eluted. The process was continued with H_2O until ca. 35 ml had been eluted, then the column flow was stopped. Eluate was diluted to the desired volume, stoppered, mixed, and kept in a refrigerator.

Duplicate 5 ml eluates or standard solutions were pipetted into 50 ml Erlenmeyer flasks, then 10 ml of 0.1 N HCl was added and the solution mixed. To each flask, 3 ml of 1 N NaOH was added and again mixed. Within 5 min, 1 ml of o-phthalicdicarboxaldehyde (OPT) solution was added and quickly mixed. After exactly 4 min, 3 ml of 3.57 N H_3PO_4 was added and quickly mixed. Within 1.5 hr, fluorescence intensity (I) of eluate and standard solutions was recorded using a model 450 digital fluorometer at excitation

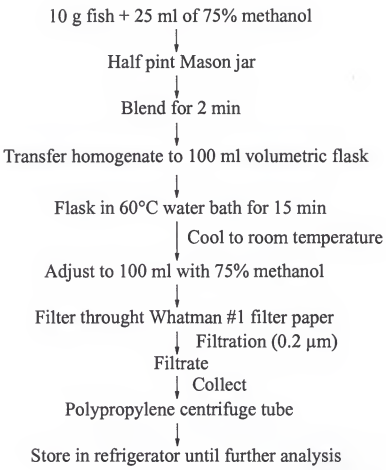


Figure 4. Schematic diagram for extraction of biogenic amines from fish fillets

wavelength of 350 nm (filter NB360) and emission wavelength of 444 nm (filter NB440). The fluorescence intensity of histamine standard solutions was used to prepare a standard curve. From this standard curve, the concentrations of histamine in fish samples were calculated. The AOAC procedure is summarized in Figure 5.

Histamine Analysis Using Gas Chromatography (GC)

The GC method used for analysis of histamine in fish is described in the biogenic amine analysis section.

Biogenic Amine Analysis

Extraction of Biogenic Amines from Mahi-mahi and Tuna Samples

The same fish extracts used for CE analysis of histamine from mahi-mahi and tuna were used for GC analysis of biogenic amines.

Preparation of Biogenic Amine Standard Solutions

Biogenic amine stock solutions (1 mg/ml) were prepared by dissolving 183 mg of putrescine dihydrochloride, 171 mg of cadaverine dihydrochloride, 166 mg of histamine dihydrochloride, 175 mg of spermidine trihydrochloride, and 172 mg of spermine tetrahydrochloride (Sigma) into respective 100 ml volumetric flasks with 0.1 N HCl. Five biogenic amine standard mixtures were prepared by adding a specified volume of each biogenic amine stock solution (Table A-1) into a 10 ml volumetric flask and then diluting to the volume with 0.1 N HCl.

Step 1. Sample Clean up

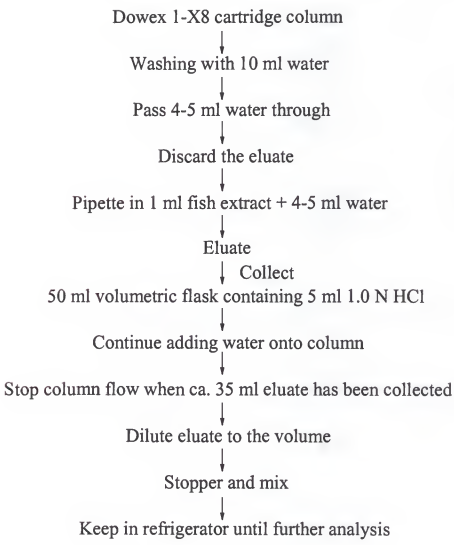


Figure 5. Schematic diagram for histamine analysis using AOAC fluorometric method

Step 2. Fluorometric Analysis

Duplicate 5 ml eluates or standard solutions

↓

50 ml Erlenmeyer flask

↓

Add 10 ml of 0.1 N HCl and mix

↓

Add 3 ml of 1 N NaOH and mix

↓

Within 5 min, pipette in 1 ml OPT solution and mix

↓

After exactly 4 min, add 3 ml of 3.75 N phosphoric acid and mix

↓

Record fluorescence intensity at excitation 350 nm (filter NB360) and emission 444 nm (filter NB 440) using model 450 digital fluorometer

OPT: o-phthalicdicarboxaldehyde

Figure 5. Continued

Derivatization of Biogenic Amines for GC Analysis

The method of Staruszkiewicz and Bond (1981) was modified for derivatization of biogenic amines from mahi-mahi samples. To each 10 ml of fish extraction solution (or 1 ml biogenic amine standard mixture solution) in 50 or 100 ml round-bottom flask, 0.5 ml of 1 N HCl was added. This was evaporated to dryness on a rotary evaporator at 50°C. One ml of 30% ethyl acetate in toluene and 300 µl of pentafluoropropionic (PFP) anhydride (Sigma) were added. The flask was stoppered, the residue mixed, and then heated at 50°C for 30 min. The solution was swirled at least once during this time. After 30 min, the solvent and reagent were evaporated under nitrogen at 50°C. The residue was dissolved in 1 ml of 30% ethyl acetate in toluene and stored in the freezer until GC analysis. All samples were filtered through a 0.2 µm nylon acrodisc 13 syringe filter (Fisher Scientific) before GC injection (Figure 6).

GC Determination of Biogenic Amines

A Perkin Elmer 8500 gas chromatograph with flame ionization detector (FID) was used for amine analysis. Separations were achieved using a 15 m x 0.32 mm DB-1 column (J&W Scientific, Folsom, CA) with a film thickness of 3 µm which was fitted with a fused silica 1 m x 0.32 mm untreated guard column (Supelco, Bellefonte, PA). Optimal analytical conditions were as follows: injection port temperature, 300°C; detector temperature, 325°C; injection volume, 1 µl; column flow, 4 ml/min; split on at 0.1 min after injection. The inlet was operated at a constant flow mode at a helium pressure of 8.0 psi. Temperature programming used for mahi-mahi samples was carried out at initial temperature, 140°C (held 0 min), increased 5°C/min to 178°C (held 0 min), and ramped, 10°C/min to a final

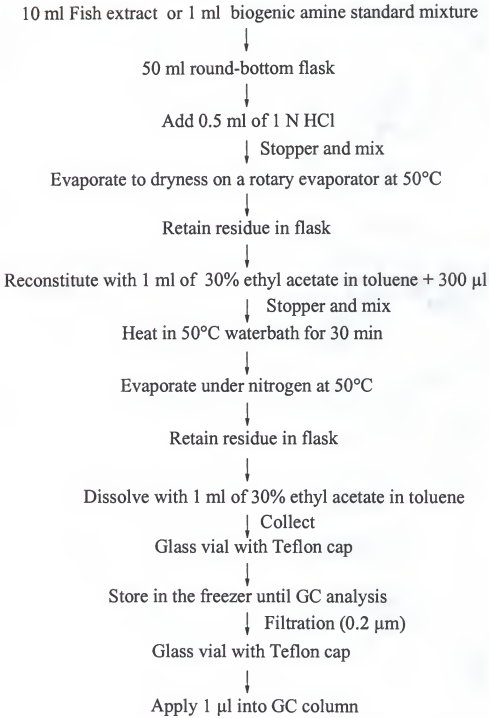


Figure 6. Schematic diagram for derivatization of biogenic amines for GC analysis

temperature, 300°C (held 5 min). For tuna samples, the temperature programming was set at 150°C at 0 min, increased 1.5°C/min to 156°C, then held for 3 min; increased 5°C/min to 161°C, held for 0 min, and raised 20°C/min to 300°C where it was held for 5 min. A H_2 pressure of 12 psi and air pressure of 22 psi were used for the FID detector.

A set of biogenic amine standard mixtures were analyzed together with test samples (blank, spiked, and stored). During analysis, a standard solution was also injected intermittently with test samples to check chromatographic consistency. Each sample was injected twice. Peak heights of each biogenic amine (putrescine, cadaverine, histamine, spermidine, and spermine) from standard mixture solutions (Table A-1) were used to prepare standard curves for each amine. From these standard curves, the amine concentrations for mahi-mahi and tuna were calculated. At the end of each experiment, the GC injector liner and septum were replaced and the column was heated overnight at 250°C.

Sensory Evaluation of Fish

Sensory Evaluation of Salmon Fillets Stored at -20°, 4°, and 10°C

On each day of testing (day 0, 3, 5, 7, 9, and 14), the fish samples from each temperature group were prepared for quality evaluation. Ten trained panelists wearing gloves were solicited to evaluate the salmon fillets by touching, smelling, and visual observance at the samples, describing whether differences occurred between samples, and commenting on specific sensory attributes, such as color and odor. Panelists also used descriptive analysis to identify the characteristics that distinguish the samples and rate the intensity of important sensory attributes. The protocols and evaluations described in the

National Marine Fisheries Service (NMFS) Fishery Products Inspection Manual (Section 1, Chapter 18, Part II, August 25, 1975) were followed.

Appendix Tables A-2 and A-3 are the evaluation sheets used by the panelists. They assessed and recorded the quality of each test sample (1-4) at different storage temperatures (A-C). Items included in Table A-2 for sensory evaluation were appearance defects, discoloration, and formation of odor. Quadruplicate samples were used for each storage temperature. The tests with salmon were conducted twice. The degree of abnormality (or defect) was categorized into slight, moderate, and excessive. Table A-3 is the score sheet used to assess the grades (A, B, and C) of each test sample at each temperature over the 14 days of storage. The fillets were considered spoiled when they had a strong discoloration and off-odor, and were unfit for human consumption.

According to the Regulations Governing Processed Fishery Products and U.S. Standards for Grades of Fishery Products (NMFS, 1975), U.S. Grade A fish fillets shall (1) possess good flavor and odor characteristic of the species and (2) comply with the limits for defects for U.S. Grade A quality as outlined in §263.104. U.S. Grade B fish fillets shall (1) possess reasonably good flavor and odor characteristic of the species and (2) comply with the limits for defects for U.S. Grade B quality in accordance with §263.104. U.S. Grade C fish fillets shall (1) possess minimal acceptable flavor and odor characteristic of the species with no objectionable off-flavors or off-odors and (2) comply with the limits for defects for U.S. Grade C quality in accordance with §263.104.

Sensory Evaluation of Salmon or Mahi-mahi Fillets Stored at 1.7°, 7.2°, and 12.8°C

A 10-point sensory scale was used for judging progressive changes in the quality attributes of salmon or mahi-mahi fillets at each sampling interval (day 0, 1, 3, and 5). Experienced judges wore gloves and evaluated the quality of fish samples by touching, smelling, visually observing, and describing any differences occurring between each temperature group. Meat color, gapping, texture, odor, and general appearance (Table A-4) were used to comment on specific differences between fish samples. Descriptive analysis was used to identify the characteristics that distinguish the samples and to develop an intensity rating of the important sensory attributes. Appendix Table A-5 is the evaluation sheet used by panelists to assess and record the quality of each test sample (1-6) at different storage temperatures (A-E).

Sensory Evaluation of Tuna Fillets Stored at 0°, 4°, 10°, and 22°C

A 10-point sensory scale was used for judging progressive changes in the quality attributes of tuna fillets at each sampling interval (day 0, 1, 3, 5, and 9). Experienced judges wore gloves and evaluated the quality of fish samples by touching, smelling, and visually observing. They described any differences occurring between each temperature group. Items included for sensory evaluation were appearance defects, discoloration, texture, and formation of odor (Table A-6). Descriptive analysis was used to identify the characteristics that distinguished the samples and to develop an intensity rating of the important sensory attributes. The degree of abnormality (or defect) was categorized into slight, moderate, and excessive. Appendix Table A-7 is the evaluation sheet used by panelists to assess and record the quality of each test sample (1-5) at different storage temperatures (A-D).

Determination of Fish Fillet Freshness Using AromaScan

Portions (10 g) of salmon, mahi-mahi, or tuna were placed in an analysis bag. The bag was evacuated and then filled with carbon-filtered air. The absolute humidity in the bag was 9 g/m^3 for salmon and 5 g/m^3 for both mahi-mahi and tuna, while the reference humidity was 15 g/m^3 for salmon, 10 g/m^3 for mahi-mahi, and 8 g/m^3 for tuna. The head space of the sample bag was allowed to equilibrate at 35°C for 10 min prior to analysis. A 2 min analysis time was performed to collect data. Prior to analysis, the polymer sensors were allowed to react with reference air (dried by silica gel to about 15 g/m^3) for 30 sec. Carbon-filtered ambient air was used as the reference air. After each analysis, the sensors were washed (1 min) with the head space from a wash bottle filled with 2% isopropanol and then allowed to react with reference air for 2.5 min before analyzing the next sample. Data for each sample were collected from a 60-second slice between 60 and 120 seconds of the total analysis cycle. Computer mapping was performed for all samples at each time-temperature storage condition using AromaScan A32S Windows Software V.1.3.

Statistical Analysis

Statistical analysis was performed using pairwise correlation analysis to determine the relationships between the production of biogenic amines in each test species and the results of sensory evaluation, as well as the results of AromaScan and bacterial counts. Pairwise correlation analysis was also used for determining the correlation between different histamine analytical methods. Microsoft Excel for Windows 95 Version 7.0 (Microsoft

Corporation, 1995) was used to calculate the mean and standard deviation of microbial, sensory, and amine data.

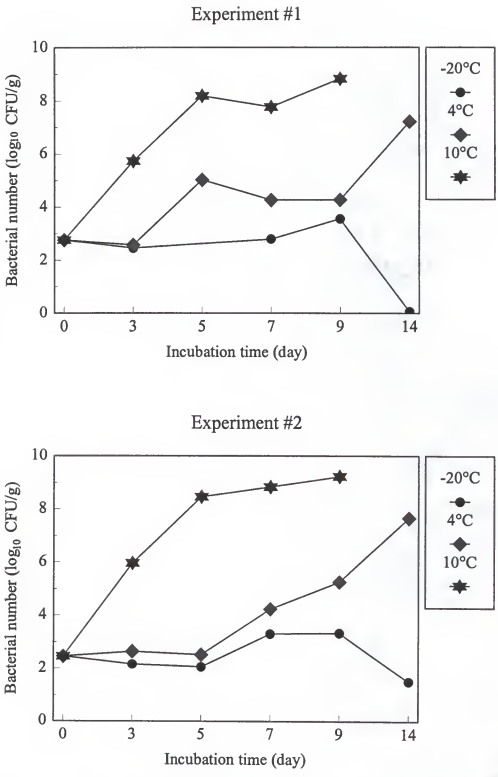
Multidimensional data were generated when a sample aroma was analyzed on AromaScan's unique 32 polymer sensor array. Sammon Mapping technique was the statistical method used for AromaScan mapping. This technique reduces the multi-dimensional original pattern space to a two- or three-dimensional pattern configuration (AromaMap) in order to visualize an easy application to gas and odor discrimination without the loss of pattern data sets. The reduced dimensional data presented in the AromaMap allowed for visual comparison of sample differences or similarities (Sammon, 1969). Multiple discriminant analysis was also used to process the AromaScan data. Multiple discriminant analysis is a statistical method that enables the reduction of multi-dimensional data into two or three dimensions, which can be viewed in a single plot (Huberty, 1975; Klecka, 1980; Tatsuoka, 1970; Tatsuoka, 1976). Significant differences between samples are shown by the spatial separation between the clusters.

CHAPTER 4
RESULTS AND DISCUSSION

Salmon Fillets Stored at -20°, 4°, and 10°C

Microbial Analysis

Stored salmon fillets showed a time- and temperature-related increases in bacterial loads (Figure 7). Highest bacterial numbers were found in samples stored at 10°C, while frozen storage showed a decrease in numbers at 14 days. Bacterial counts for salmon fillets stored at 10°C increased dramatically from 2.73 to 9.06 $\log_{10}$ CFU/g on day 9. For those samples stored at 4°C, the bacterial counts increased gradually from 2.73 to 7.46 $\log_{10}$ CFU/g over 14 days. For fillets stored at -20°C, the bacterial counts slowly increased to 3.62 $\log_{10}$ CFU/g on day 9 and then decreased to about one $\log_{10}$ CFU/g on day 14. In general, as the bacterial counts on these fillets reached 7 $\log_{10}$ CFU/g or higher, spoilage characteristics, such as slime formation and occurrence of odor and yellowish color, were noticed. The International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends that 3 of 5 samples should have aerobic plate counts (APCs) of less than 5×10^5 and no sample should have an APC of more than 10^7 CFU/g. Therefore, using this microbiological criteria, fish should be considered as unacceptable and rejected from consumption when the bacterial counts reached 7 $\log_{10}$ CFU/g or higher.



Each point represents the mean of four samples.

Figure 7. Time-related changes in bacterial numbers on salmon fillets stored at -20°, 4°, and 10°C

Identification of Histamine-Producing Bacteria in Stored Salmon Fillets

Pseudomonas putrefaciens and *Moraxella* spp. were identified through the use of Niven's differential agar, API 20E strips, and API OF and M media to be the bacterial species responsible for histamine production on positive salmon fillets. Middlebrooks et al. (1988) also isolated *Pseudomonas putrefaciens* from Spanish mackerel that showed histidine decarboxylase activity. They reported this to be the most frequently encountered species isolated from fish decomposed at 30°C. *Pseudomonas* spp. are widely distributed in nature and may have already been associated with the fish at the time of capture or subsequent handling prior to the storage experiments. *Moraxella* spp. has been isolated from a variety of marine fish by Okuzumi et al. (1981). However, they reported that these *Moraxella* spp. did not show histidine decarboxylase activity in infusion broth incubated at 5°C. Thus, the two histamine-producing bacterial species isolated from salmon fillets are the common flora associated with fish or their living environment.

Determination of Histamine by Capillary Electrophoresis

The CE electropherograms of histamine standard and muscle extracts of blank, histamine-spiked, and tested salmon fillets did not appear to have interference with the histamine peak (Figure 8). The migration time of pure histamine was 1.7 min under a constant voltage of 10 kV using a 24 cm x 25 μm capillary cartridge. Very high linear relationships ($r^2_{Ave} > 0.999$) occurred between the peak areas and histamine concentrations over 0.5 to 100 ppm in standard solutions. r^2_{Ave} is an approximate value of the coefficient of determination. The value of r^2_{Ave} is only approximate since the data fitted were averaged across the four replicates rather than the individual values. Realizing that the approximate

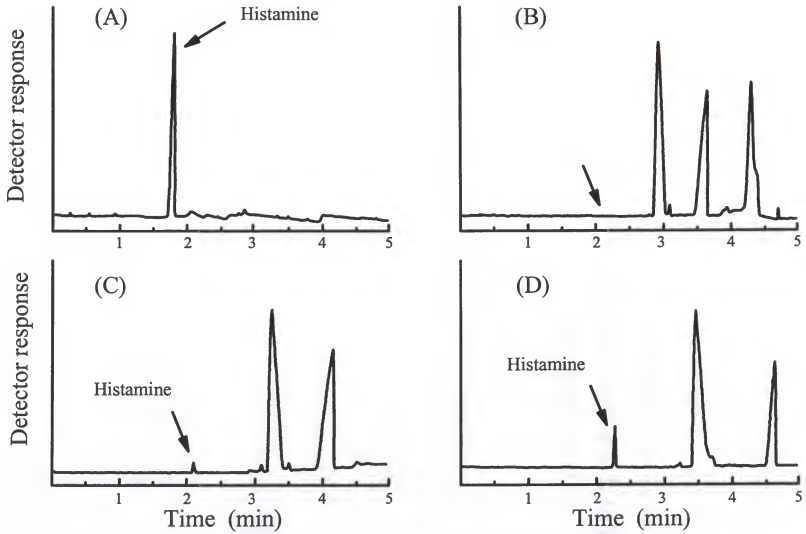


Figure 8. Typical electropherograms of (A) histamine standard solution, (B) muscle extracts of blank salmon, (C) treated salmon fillets, and (D) histamine-spiked salmon

values of r^2_{Ave} are inflated slightly because the models were fitted to averages and not individual observations (Cornell and Berger, 1987). A 90.9% recovery rate was achieved from histamine-spiked salmon fillets over the concentration range of 25 to 250 ppm.

Histamine was detected from the two experiments in two of the eight fillets stored at 4°C and four fillets stored at 10°C for seven days, and all eight fillets stored at 10°C for nine days. Histamine contents ranged from <0.5 to 54.7 ppm in positive fillets (Table 1). However, histamine was not detected in any of the fillets stored at -20° or 4°C for 14 days. No correction for recovery loss was made with these data. A wide range of inter-fillet differences in histamine content makes interpretation of the results difficult. However, salmon fillets stored at 10°C for 9 days tended to have higher histamine content than those stored for 7 days at 4° or 10°C (Table 1).

Salmon has seldom been reported as a vehicle of scombroid poisoning in the United States; between 1978-1986, only two outbreaks were reported involving salmon (*Oncorhynchus*) (Ahmed, 1991). Mietz and Karmas (1978) detected histamine at 0.34 - 5.44 ppm in salmon steak obtained from coastal areas of Alaska. Therefore, the low levels of histamine found in salmon fillets were expected.

Murray et al. (1982) showed that the level of histamine does not increase to over 50 ppm when fresh mackerel was allowed to spoil in ice and become unfit for consumption. The low histamine content found in our salmon fillets after 7 days of storage at 4°C and no histamine in fish stored at -20°C for 7 and 9 days can be explained by the inhibitory effect of low temperature (< 7°C) on histamine-producing bacteria (Behling and Taylor, 1982). It is interesting to note that the bacterial loads reached the levels ≥ 7 $\log_{10}$ CFU/g on salmon

Table 1. Histamine content (ppm) in salmon fillets stored at different temperatures for 7 and 9 days

Day	Temperature	Experiment #1				Experiment #2			
		Fillet sample				Fillet sample			
		I	II	III	IV	I	II	III	IV
7	- 20°C	ND[1]	ND	ND	ND	ND	ND	ND	ND
	4°C	9.6	ND	15.7	ND	ND	ND	ND	ND
	10°C	17.5	3.7	3.7	7.3	ND	ND	ND	ND
9	- 20°C	ND	ND	ND	ND	ND	ND	ND	ND
	4°C	ND	ND	ND	ND	ND	ND	ND	ND
	10°C	54.7	7.1	23.5	20.2	9.0	< 0.5	3.1	15.3

[1] ND = not detected.

fillets stored at 10°C when histamine was detected. Ienistea (1973) indicated that histamine production is not related to total bacterial loads, but is influenced by the number of bacteria able to synthesize histidine decarboxylase. These results indicate that histamine production in salmon fillets is not always associated with sensory changes in smell/odor of the fish.

Sensory Evaluation of Salmon Fillets Stored at -20°, 4°, and 10°C

Fresh salmon fillets had a firm texture with typical salmon-orange color and no off-odor. Color and odors of test fillets changed as storage time and temperature increased. Panelists noted the disappearance of fresh fishy (seaweedy) odor whereas raw odors and sourness increased during storage, especially in the group stored at 10°C. The growth of lactic acid bacteria during storage resulted in the souring of fresh fish (Lindsay et al., 1987).

Panelists rated all salmon fillets as grade A products on day 0 (Table 2). The grade of salmon decreased to B or lower C after storage at different temperatures for 5 days. Sensory panelists rated the 10°C samples as grade C products and unacceptable after day 3. Samples stored at 4°C began to show deterioration by day 7, and were all considered unacceptable by day 14. Salmon fillets stored at -20°C exhibited only slight fish odor and slight discoloration even after 14 days. These fillets were rated acceptable throughout the study and served as a control. Sensory evaluation by the panel showed that some of the fillets stored at 10°C started to release excessive fishy odor after 5 days. Some of the fillets stored at 4°C started to exhibit moderate discoloration and raw odor after 7 days.

All fish samples were rejected for consumption when they were rated as grade C products. Good correlation was found between sensory grading of salmon fillets and their bacterial counts. It was noted that when the bacterial number reaches 7 $\log_{10}$ CFU/g or

Table 2. Quality grading of salmon fillets stored at different temperatures for up to 14 days

Temperature	Day	Experiment #1				Experiment #2			
		Fillet sample				Fillet sample			
		I	II	III	IV	I	II	III	IV
	0	A[1]	A	A	A	A	A	A	A
- 20°C	3	B	B	B	A	A	A	A	A
	5	B	B	B	B	B	B	B	B
	7	B	B	B	B	B	B	B	B
	9	B	B	B	B	B	B	B	B
	14	B	B	B	B	B	B	B	B
4°C	3	B	B	B	B	B	B	B	B
	5	B	B	B	B	B	B	B	B
	7	B	C	B	C	B	B	B	B
	9	B	C	B	B	B	B	B	B
	14	C	C	C	C	C	B	C	C
10°C	3	C	C	C	C	C	C	C	C
	5	C	C	C	C	C	C	C	C
	7	C	C	C	C	C	C	C	C
	9	C	C	C	C	C	C	C	C

[1] Each grade represents the mean of 10 judgements.

higher, the fish was considered spoiled by the panelists and rated as grade C products, not suitable for consumption (Figure 9). Fish samples considered as acceptable (grade A or B) had a bacterial load of 3 $\log_{10}$ CFU/g or lower. Thus, the microbial counts played an important role in spoilage of fresh salmon fillets, and sensory evaluation remains a good test for fish quality.

AromaScan Analysis of Salmon

Time-related changes in sensory quality of salmon fillets stored at -20°, 4°, and 10°C are shown in Figure 10. The mappings of the three temperature groups were separated from that of day 0 samples in a time-related fashion. The AromaMaps for the three temperature groups did not separate from each other by day 3 of storage, although they separated from the cluster of day 0 controls. The 10°C samples started to show a population mapping separate from those of the other two groups (-20° and 4°C) by day 5. As the storage time increased to 7 days or longer, the 10°C population was further separated from those of the other two groups. By day 9, the mappings from all three temperature groups and fresh (day 0) were separated from each other.

Results for AromaScan analysis of salmon fillets stored at -20°, 4°, and 10°C for five different time intervals (days 3, 5, 7, 9, and 14) are shown in Figure 11. The AromaMap of day 3 samples did not separate from that of day 0 samples at 4°C. However, day 3 samples stored at -20° and 10°C separated from day 0 samples, as well as others stored at these temperatures for the longer times (5, 7, 9, and 14 days). The mappings of day 5, 7, and 9 samples did not separate from each other for all three temperatures, but were separated from that of fresh samples.

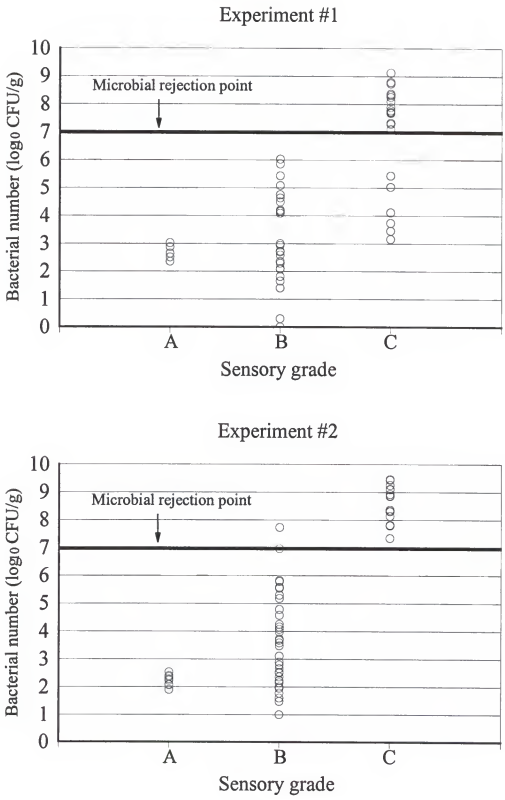


Figure 9. Correlation of sensory grading with bacterial counts in salmon fillets stored at -20°, 4°, and 10°C

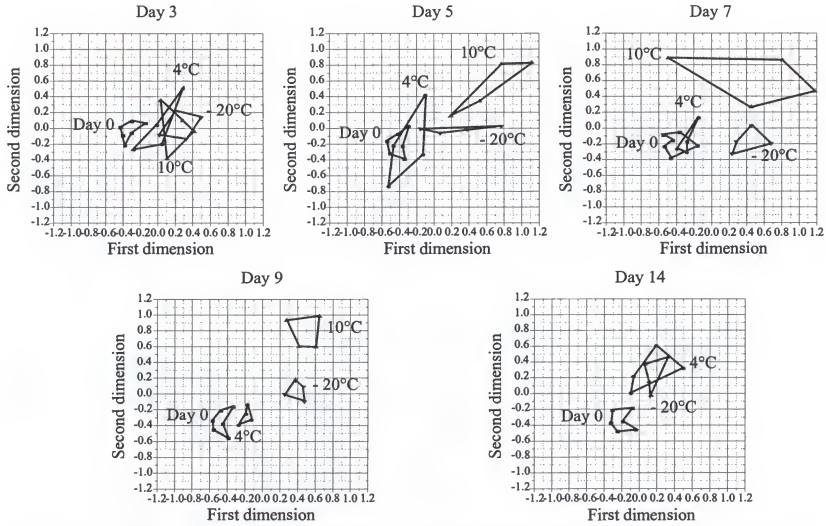


Figure 10. Comparison of AromaMaps for salmon fillets stored at different temperatures on day 3, 5, 7, 9, and 14 of storage. All fillets were compared to day 0 controls.

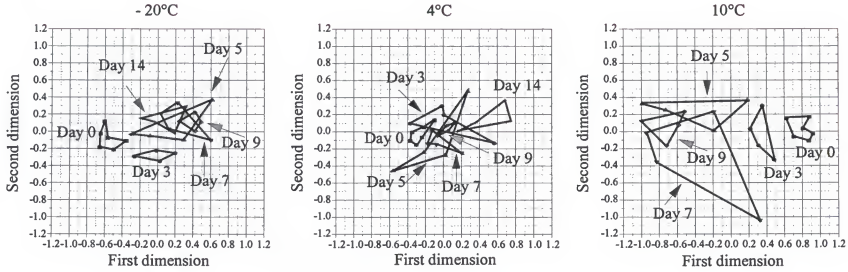


Figure 11. Comparison of AromaMaps for salmon fillets stored at - 20°, 4°, and 10°C for various time periods. All fillets were compared to day 0 controls.

Multiple discriminant analysis (in Statistica software V 4.5, StatSoft, Inc., 1993) was also used to process data obtained from the AromaScan analyses and these data were compared with those for sensory and microbial results. Due to the limitation of this software, only the data from16 sensors were used. After comparing all the data from 32 sensors, the data obtained from the sensors that showed a higher correlation for odor detection were removed from analysis, because they had similar response to the odor profile of fish samples. Only the data from the other 16 sensors showing low correlations (better detection of odor discrepancy) were used. In discriminant analysis, the X and Y functions account for differences between different samples rather than replicate to replicate differences (system or method parameters).

A canonical discriminant graph of salmon fillets stored at -20° and 4°C (Figure 12) shows that AromaScan can be used to differentiate salmon fillets stored at different temperatures by storage days. As the storage time increased, fish fillets in different clusters separated even further. A very good correlation was found between the results of AromaScan and microbial analysis. The clusters of salmon fillets stored at different temperatures with different microbial loads separated from each other in the canonical discriminant graphs (Figure 13). The clusters with the closer bacterial numbers were closer to each other in the graph. A high correlation was also found between the results of AromaScan and sensory analysis. The test results of AromaScan can be used to predict the grade of salmon fillets stored at 4° and 10°C (Figure 14). Compared to the clusters of salmon fillets stored at 10°C, the cluster of grade B salmon fillets stored at 4°C was closer to that of grade C fillets. The cluster of grade A fillets was farther away from those of grades

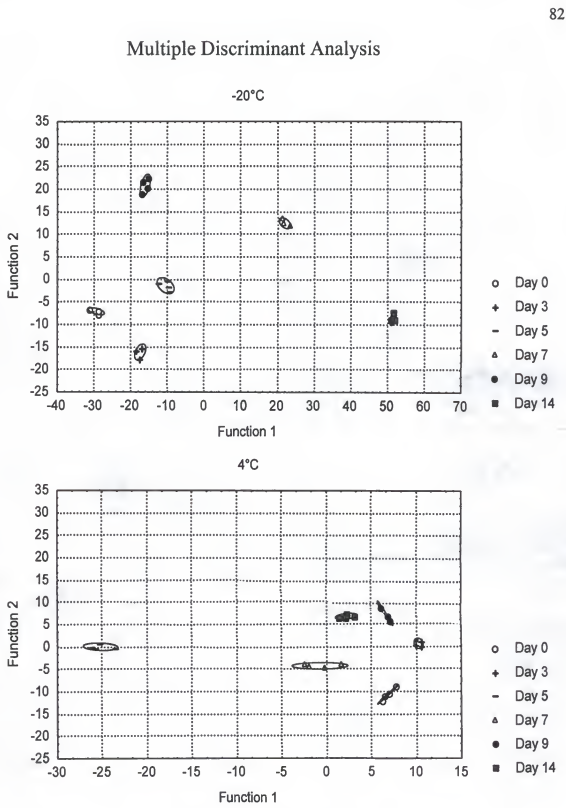


Figure 12. Canonical discriminant graphs for salmon fillets stored at -20° and 4°C for up to 14 days

Multiple Discriminant Analysis

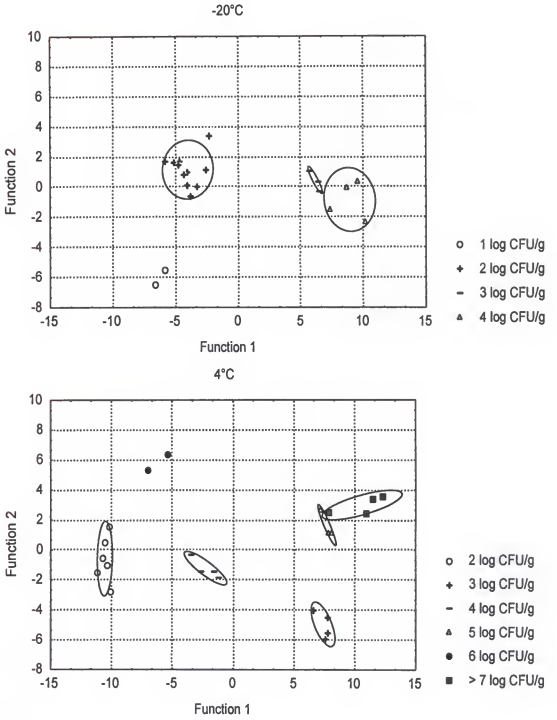


Figure 13. Correlation of AromaScan analyses with the bacterial loads in salmon fillets stored at -20° and 4°C

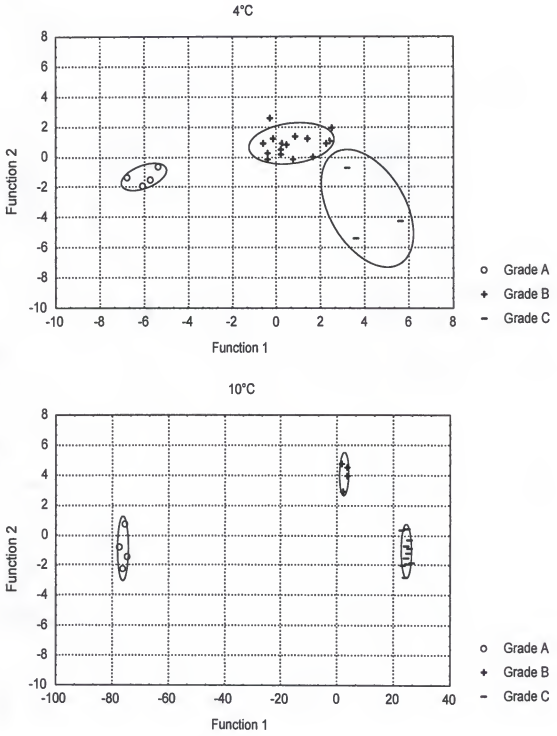


Figure 14. Correlation of AromaScan analyses with sensory grading for salmon fillets stored at 4° and 10°C

B and C in both temperature groups. This indicated that grade A fillets had an odor far different from those of grades B and C fillets. This result indicated that AromaScan can be used for quality and freshness evaluation of salmon.

Salmon Fillets Stored at 1.7°, 7.2°, and 12.8°C

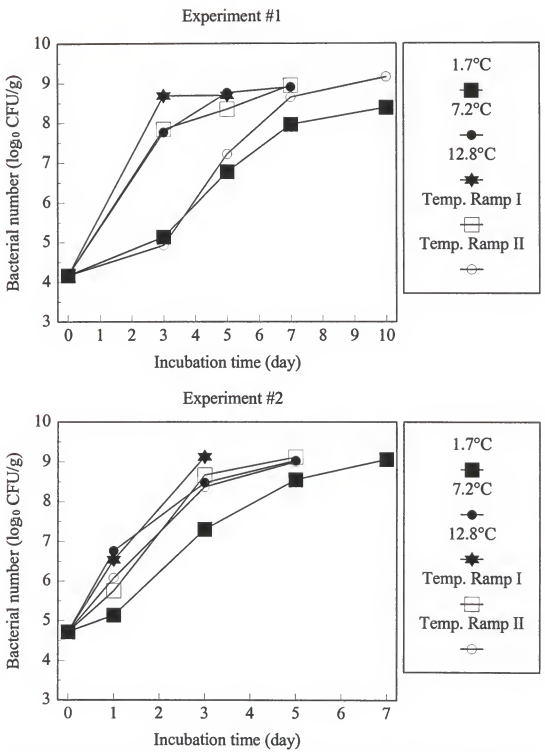
Bacterial Enumeration

Time-related changes in bacterial growth for salmon stored at 1.7°, 7.2°, and 12.8°C are shown in Figure 15. Bacterial counts for samples stored at 1.7°C increased gradually from 4.45 $\log_{10}$ CFU/g to 8.52 $\log_{10}$ CFU/g over 7 days. For fillets stored at 7.2°C, bacterial counts increased to 8.9 $\log_{10}$ CFU/g on day 5. The bacterial counts in samples stored at 12.8°C increased dramatically to 8.91 $\log_{10}$ CFU/g after 3 days. Bacterial counts for the two temperature ramp groups (Temp. Ramp I and II) were similar to that of the 7.2°C group.

Sensory Evaluation of Salmon Fillets

There was no discernible difference in the sensory attributes of raw fillets from each individual box upon arrival at Gainesville . All fillets appeared in excellent condition and all VITSAB's visual time-temperature integrators were 'green'. Likewise, all fillets within each box appeared to be similar on arrival in Gainesville.

Ratings from 1 to 4 were considered most desirable or grade A product. Ratings from 4 to 6 were considered acceptable, but less desirable or grade B product. Ratings greater than 6 were considered unacceptable or grade C product. Sensory ratings for general appearance, meat color, and odor were the most accurate for predicting storage consequences. Sensory evaluation by the panel showed that some of the salmon fillets stored at 12.8°C started to



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Each point represents the mean of six samples.

Figure 15. Time-related changes in bacterial loads for salmon fillets stored at 1.7°, 7.2°, 12.8°C, and temperature ramp I and II

release excessive fish odor after day 3 (Table 3). Some of the fillets stored at 7.2°C started to exhibit moderate discoloration and slight fish odor after 3 days. Fish fillets stored at 1.7°C exhibited only slight fish odor and moderate discoloration even after 7 days. Both groups of salmon fillets that were stored at 1.7°C and then exposed to 12.8°C for 24 hr showed intense odor and discoloration similar to that stored at 7.2°C for 3 days. The salmon fillets stored at 12.8°C were deemed unacceptable after 3 days (Figure 16). Both groups of salmon fillets that were stored at 1.7°C and then exposed to 12.8°C for 24 hr and the salmon fillets stored at 7.2°C were considered unacceptable after 5 days. The salmon fillets stored at 1.7°C remained acceptable even after 7 days of storage.

AromaScan Analysis of Salmon Stored at 1.7°, 7.2°, and 12.8°C

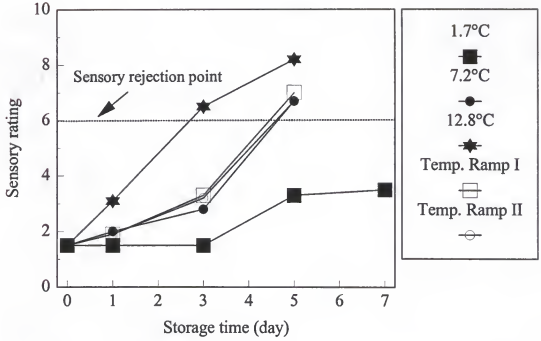
Results of AromaScan analysis of salmon stored at 1.7°, 7.2°, and 12.8°C for 3 time intervals (day 1, 3, and 5) are shown in Figure 17. Salmon fillets stored at all temperatures did not separate from each other on day 1. However, their mappings were separated from the day 0 controls. A time-related separation was found among samples stored at different temperatures. The mappings of samples stored at different temperatures separated further on day 5 than those on day 3.

Analysis by AromaScan of the sensory quality of salmon fillets stored at different temperatures (Figure 18) showed that mappings for the five groups were separated from that of the day 0 samples in a time-related fashion. Samples stored at 1.7°C for different days did not separate from each other. A time-related separation was found in samples stored at 7.2° and 12.8°C; their maps continued to separate further as storage time increased. Mappings of samples stored at the two temperature ramp groups were similar to those for the 7.2°C

Table 3. Changes in sensory rating by a sensory panel for salmon fillets stored at different temperatures

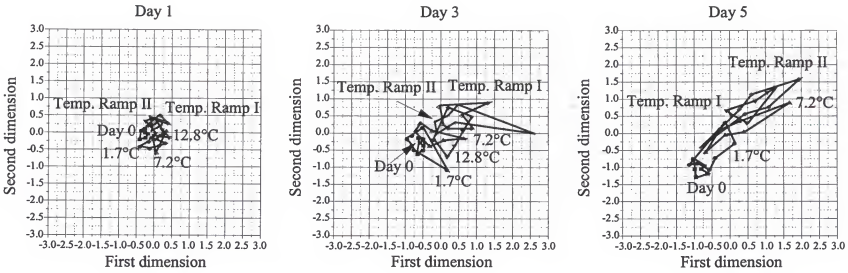
Day	Temperature	Parameters					Mean ± S.D.
		Appearance	Color	Gap	Texture	Odor	
0		1.5[1]	1.5	1.5	1.5	1.5	1.5 ± 0.0
1	1.7°C	1.5	1.5	1.5	1.5	1.5	1.5 ± 0.0
	7.2°C	2.0	2.5	1.7	1.8	1.8	2.0 ± 0.3
	12.8°C	3.5	3.6	2.2	2.5	3.6	3.1 ± 0.6
	Temp. Ramp I[2]	1.9	2.6	1.6	1.8	1.8	1.9 ± 0.4
	Temp. Ramp II[3]	1.8	2.6	1.6	1.8	1.8	1.9 ± 0.4
3	1.7°C	1.5	1.5	1.5	1.5	1.5	1.5 ±0.0
	7.2°C	3.5	4.5	2.0	2.0	2.0	2.8 ± 1.0
	12.8°C	7.5	8.0	3.9	5.0	8.0	6.5 ± 1.7
	Temp. Ramp I	4.5	4.5	2.0	2.0	3.5	3.3 ± 1.3
	Temp. Ramp II	3.8	4.7	1.9	2.0	3.5	3.2 ± 1.2
5	1.7°C	3.5	4.5	2.0	3.5	3.0	3.3 ± 0.8
	7.2°C	8.5	8.5	2.5	6.0	8.0	6.7 ± 2.3
	12.8°C	9.5	9.5	5.0	7.5	9.5	8.2 ± 1.8
	Temp. Ramp I	8.9	8.7	2.6	6.5	8.2	7.0 ± 2.6
	Temp. Ramp II	8.5	8.4	2.5	6.0	8.0	6.7 ± 2.5
7	1.7°C	4.5	4.5	2.0	3.5	3.0	3.5 ± 0.9

[1] Each rating represents the mean of 10 judgements.
[2] Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
[3] Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.



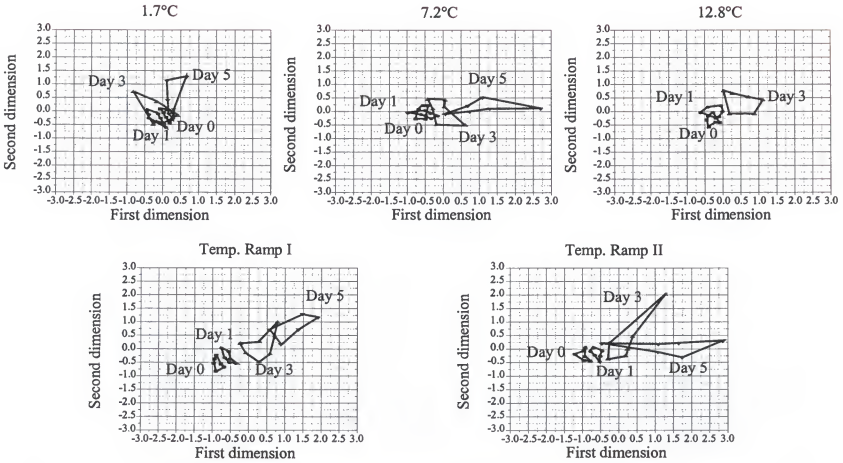
Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 16. Sensory rating of salmon fillets stored at different temperatures for up to 7 days



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 17. Comparison of AromaMaps for salmon fillets stored at different temperatures on day 1, 3, and 5 of storage. All fillets were compared to day 0 controls.



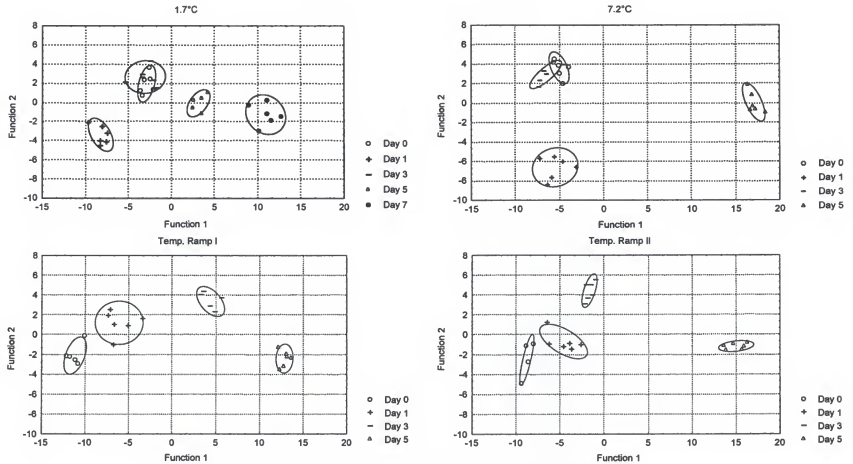
Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 18. Comparison of AromaMaps for salmon fillets stored at 1.7°, 7.2°, 12.8°C, and temperature ramp I and II for various time periods. All fillets were compared to day 0 controls.

group. Mappings for day 0 and day 1 samples were separated from each other for the two temperature ramp groups and these continued to separate from those of day 3 and 5 groups.

Multiple discriminant analysis was used to show significant differences between samples by the spatial separation between clusters. In discriminant analysis, the X and Y functions account for differences between different samples. The results showed that the odor profiles from salmon stored at the two temperature ramp groups could be differentiated by the day of storage as separate clusters (Figure 19). Canonical discriminant graphs showed that salmon samples stored at different temperatures were separated from each other in a time-related fashion. Their clusters continued to separate further as the storage time increased. The cluster for day 0 samples could not be separated from those of day 3 samples at 1.7° and 7.2°C, but could be separated for the two temperature ramp groups. Shifting the storage temperature from 1.7°C to 12.8°C for one day apparently caused changes in salmon quality as reflected by odor profile.

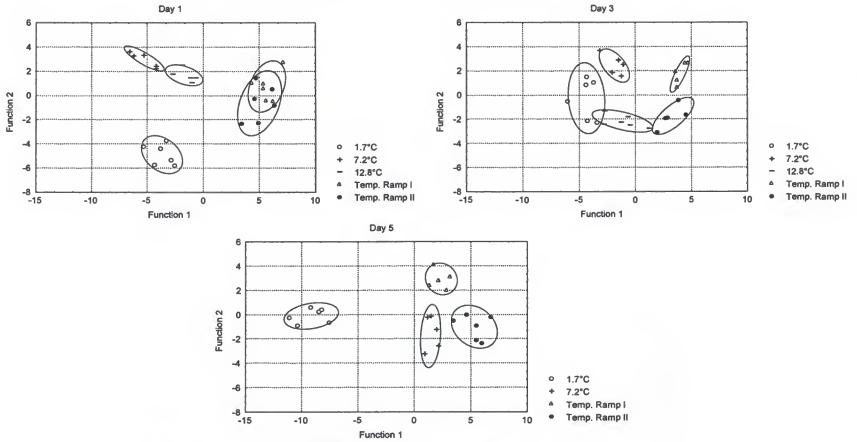
Canonical discriminant graphs (Figure 20) showed that the cluster of salmon samples stored at three different temperatures (1.7°, 7.2°, and 12.8°C) were separated from each other on day 1. However, the clusters for the two temperature ramp groups did not separate from each other until day 3. The clusters of salmon samples showed a temperature-related separation in the plots; those stored at closer temperatures had less spatial separation. The different stages of decomposition in salmon stored at different temperatures were clearly grouped into distinct regions of the plot on day 5. The cluster of salmon fillets stored at 1.7°C was farther away from those of the other temperature groups. This indicated that the AromaScan identified the odor profiles of salmon fillets stored at 1.7°C as significantly



Temp. Ramp. I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 19. Canonical discriminant graphs for salmon fillets stored at 1.7°, 7.2°C, and temperature ramp I and II for various time periods



Temp. Ramp. I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 20. Canonical discriminant graphs for salmon fillets stored at different temperatures on day 1, 3, and 5 of storage

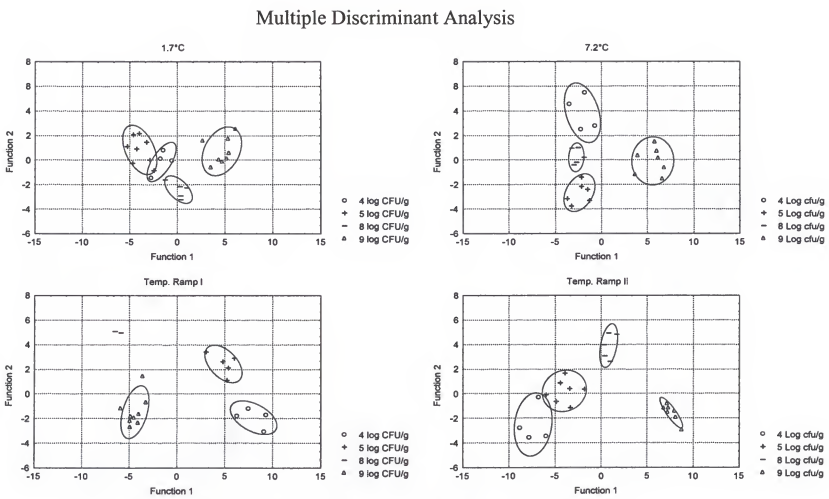
different from those of other temperature groups. Therefore, the AromaScan was capable of differentiating changes in odor profiles of salmon samples during storage.

The clusters for salmon fillets stored at different temperatures and compared to microbial loads were separated from each other in the canonical discriminant graphs (Figure 21). Clusters with close bacterial numbers were not as separated from each other. High correlation was also found between the AromaScan and sensory analysis. AromaScan can predict the grade of salmon fillets stored at the two temperature ramp groups (Figure 22). Compared to the clusters of salmon fillets stored at Temp. Ramp I, the cluster of grade B fillets stored at Temp. Ramp II was closer to that of grade A fillets. The cluster of grade C fillets was farther away from those of grade A and B samples in both temperature groups. This indicated that the grade C fillets had an odor profile far different from those of grade A and B fillets. Thus, AromaScan can be used for quality and freshness evaluation of salmon.

Mahi-mahi Fillets Stored at 1.7°, 7.2°, and 12.8°C

Bacterial Enumeration

Stored mahi-mahi fillets showed time- and temperature-related increases in bacterial loads. Those fillets stored at 7.2° and 12.8 °C showed a dramatic increase in bacterial counts, reaching > 9 $\log_{10}$ CFU/g in 3 days (Figure 23). Those fillets stored at 1.7°C did not reach this level until day 5. The changes in bacterial counts for the two temperature ramp groups (Temp. Ramp I and II) were similar to the 7.2 °C group. It was noted that, when the bacterial number reached 7 $\log_{10}$ CFU/g or higher, the fish meat was considered spoiled and rejected

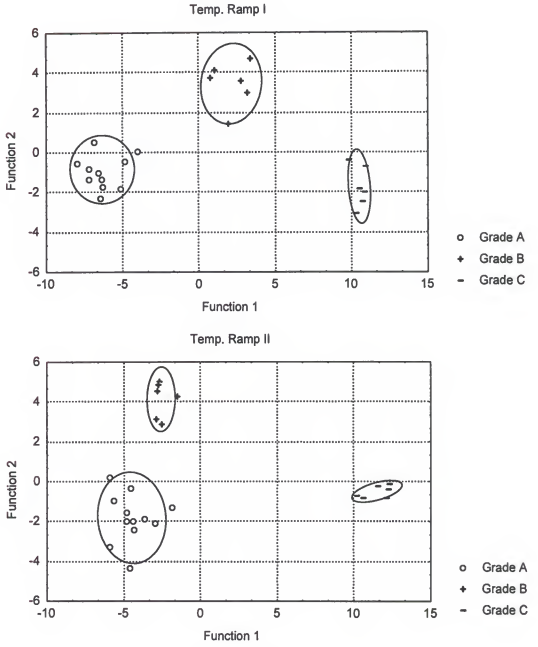


Temp. Ramp. I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

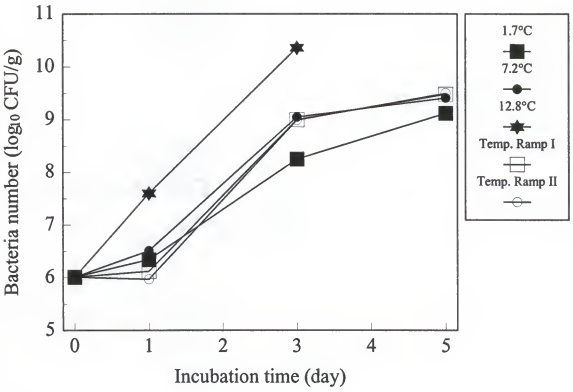
Figure 21. Correlation of AromaScan analyses with bacterial counts in salmon fillets stored at 1.7°, 7.2°C, and temperature ramp I and II

Multiple Discriminant Analysis



Temp. Ramp. I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 22. Correlation of AromaScan analyses with sensory grading for salmon fillets stored at temperature ramp I and II



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Each point represents the mean of six samples.

Figure 23. Time-related changes in bacterial loads for mahi-mahi fillets stored at 1.7°, 7.2°, 12.8°C, and temperature ramp I and II

for consumption. Mahi-mahi fillets stored at 12.8 °C were rejected by day 1 (based on the microbiological standard). Fillets stored at all other temperatures were rejected by day 3.

Capillary Electrophoresis Determination of Histamine

The CE electropherograms of a histamine standard and muscle extracts of blank, histamine-spiked, and tested mahi-mahi fillets did not appear to have interference with the histamine peak (Figure 24). The migration time of pure histamine was 2.7 min under a constant voltage of 10 kV using a 24 cm x 25 μm capillary cartridge. Very high linear relationships ($r^2_{Ave} > 0.999$) occurred with peak areas and concentrations of histamine over 0.5 to 100 ppm. Recovery rate (87.5%) was achieved from histamine-spiked mahi-mahi fillets over the concentration range of 25 to 500 ppm (Table 4). The average coefficient of variation was 4.6% for intra-assay and 7.7% for interassay with histamine standard solutions. Thus, a very good reproducibility was achieved with CE analysis of histamine in mahi-mahi extracts. The mean recovery rate of histamine from histamine-spiked mahi-mahi fillets was 98.8% over the concentration range of 25 to 500 ppm when using 75% methanol instead of 50% for extraction.

Mahi-mahi (dolphin fish), a non-scombroid fish, have been widely recognized to contain elevated levels of histamine (MMWR 1989; MMWR, 1986). Muscle of mahi-mahi, like the Scombroid fish, contain large amounts of histidine which is readily susceptible to formation of toxic levels of histamine. One scombrotoxin outbreak in 1990 caused by tainted mahi-mahi, resulted in 148 cases of foodborne diseases (MMWR, 1996).

High levels of histamine (154 to 817 ppm) were found in fish after 5 days of storage at 12.8°C (Table 5). Less than 10 ppm histamine was found in mahi-mahi fillets stored at

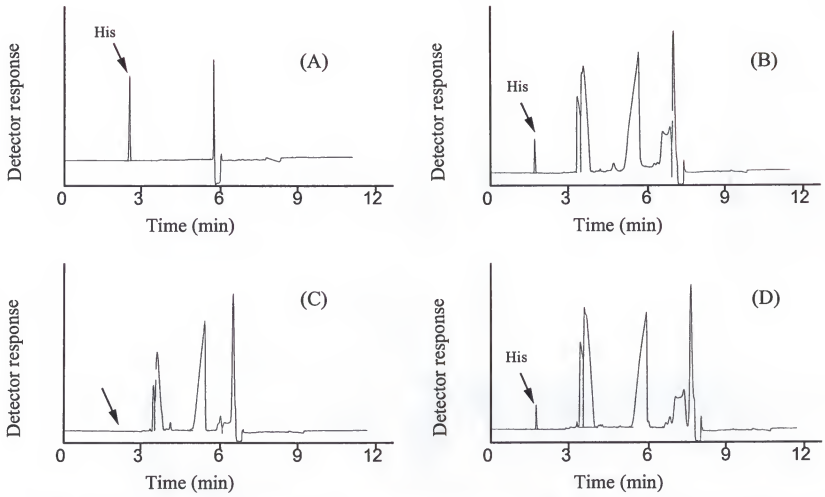


Figure 24. Typical electropherograms of (A) histamine standard solution, (B) muscle extracts of histamine-spiked mahi-mahi, (C) blank mahi-mahi, and (D) treated mahi-mahi fillets

Table 4. Recovery of histamine from fortified mahi-mahi muscle by CE method

Fortification level (ppm)	Conc. found (ppm)	Average recovery rate (% mean ± S.D., n=5)	Coefficient of variation (%)
25	24.0	96.0 ± 8.2	8.5
50	43.4	86.8 ± 7.4	8.5
125	108.9	87.1 ± 7.5	8.6
250	213.0	85.2 ± 9.7	11.4
500	411.0	82.2 ± 6.1	7.4
Mean		87.5	

Table 5. Histamine concentrations (ppm) in mahi-mahi stored at different temperatures for up to 5 days as analyzed by CE

Day	Temperature	Fillet sample						Mean ± S.D.
		I	II	III	IV	V	VI	
0		ND[1]	6.4	ND	ND	ND	ND	1.1 ± 2.6
1	1.7°C	ND	ND	ND	ND	6.3	ND	1.1 ± 2.6
	7.2°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	12.8°C	ND	13.2	ND	ND	ND	ND	2.2 ± 5.4
	Temp. Ramp I[2]	15.7	ND	16.5	ND	ND	ND	5.4 ± 8.3
	Temp. Ramp II[3]	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
3	1.7°C	ND	ND	ND	ND	5.5	ND	0.9 ± 2.2
	7.2°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	12.8°C	19.9	16.6	15.7	11.4	41.8	14.2	19.9 ± 11.1
	Temp. Ramp I	13.3	ND	ND	ND	ND	ND	2.2 ± 5.4
	Temp. Ramp II	ND	5.6	ND	ND	ND	ND	0.9 ± 2.3
5	1.7°C	ND	ND	ND	ND	8.2	ND	1.6 ± 3.7
	7.2°C	ND	4.1	4.3	8.7	15.8	0.8	5.6 ± 5.9
	12.8°C	202.4	153.8	187.1	392.3	816.5	224.2	329.4 ± 252.8
	Temp. Ramp I	25.0	ND	ND	ND	4.6	2.9	5.4 ± 9.8
	Temp. Ramp II	123.3	4.9	3.2	3.6	ND	29.9	27.5 ± 48.2

[1] ND = not detected
[2] Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
[3] Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

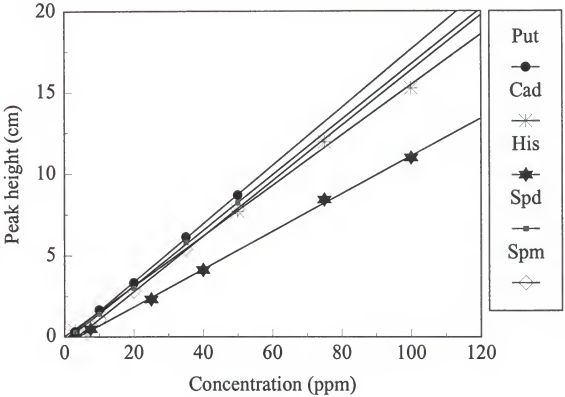
1.7 °C for 5 days. A high variation in histamine content was found among mahi-mahi fillets stored at the same temperature for the same period of time. This inconsistency in histamine content even within the same species could be due to variations in how the fish is processed and decomposed (Mietz and Karmas, 1978).

Gas Chromatography Determination of Biogenic Amines

The GC method can simultaneously determine the contents of putrescine (Put), cadaverine (Cad), histamine (His), spermidine (Spd), and spermine (Spm) in mahi-mahi fillets within 20 min following pentafluoropropionic (PFP) anhydride derivatization of methanol extracts. The retention times were 3.6, 5.1, 7.1, 11.6, and 16.5 min for Put, Cad, His, Spd, and Spm, respectively. Standard curves for these five amines were linear ($r^2_{Ave} >$ 0.99) over the selected concentration ranges (Figure 25).

Gas chromatograms of PFP derivatives of amine standards and muscle extracts of blank, amine-spiked, and tested mahi-mahi fillets did not appear to have interference with amine peaks (Figure 26). The recovery rates for the amines were 99.5, 95.8, 86.3, 93.3, and 64.9% for Put, Cad, His, Spd, and Spm, respectively. The average coefficient of variation for the intra-assay of the amine standard solution was 5.4, 4.0, 10.7, 5.2, and 6.4% for Put, Cad, His, Spd, and Spm, respectively, while it was 8.6, 14.7, 17.7, 4.0, and 7.4%, respectively, for the interassay.

High levels of histamine (158 to 764 ppm) were found in fish after 5 days of storage at 12.8°C (Table 6). Less than 10 ppm histamine was found in mahi-mahi fillets stored at 1.7°C for 5 days. Except for one sample stored at Temp. Ramp II for 5 days, the histamine



Putrescine (Put):	Y=0.1784X-0.1794	r-square=0.9996	3-50 ppm
Cadaverine (Cad):	Y=0.1546X+0.0214	r-square=0.999	3-100 ppm
Histamine (His):	Y=0.1156X-0.4549	r-square=0.999	7.5-100 ppm
Spermidine (Spd):	Y=0.1702X-0.2596	r-square=0.9992	3-50 ppm
Spermine (Spm):	Y=0.17X-0.6014	r-square=0.9999	5-50 ppm

r-square is approximate since the data fitted were averaged across the four replicate rather than the individual values.

Figure 25. Standard curves of the five biogenic amines analyzed by gas chromatography

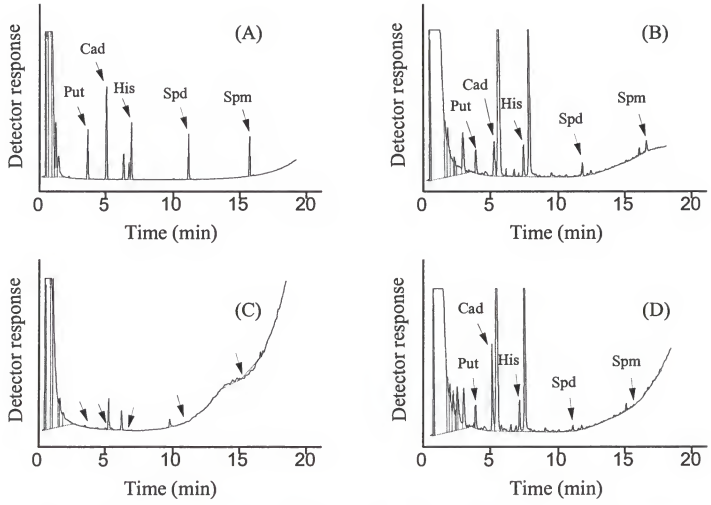


Figure 26. Gas chromatograms of PFP derivatives of (A) a biogenic amines standard mixture, (B) muscle extracts of biogenic amines-spiked mahi-mahi, (C) blank mahi-mahi, and (D) treated mahi-mahi fillets

Table 6. GC analysis of histamine (ppm) in mahi-mahi stored at different temperatures for up to 5 days

Day	Temperature	Fillet sample						Mean ± S.D.
		I	II	III	IV	V	VI	
0		ND[1]	ND	ND	ND	ND	ND	0.0 ± 0.0
1	1.7°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	7.2°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	12.8°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	Temp. Ramp I[2]	ND	ND	5.9	ND	ND	ND	1.0 ± 2.4
	Temp. Ramp II[3]	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
3	1.7°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	7.2°C	ND	ND	ND	ND	ND	ND	0.0 ± 0.0
	12.8°C	ND	ND	ND	5.7	7.8	5.8	3.2 ± 3.6
	Temp. Ramp I	13.1	ND	ND	ND	ND	ND	2.2 ± 5.4
	Temp. Ramp II	ND	5.8	ND	ND	ND	ND	1.0 ± 2.4
5	1.7°C	ND	ND	ND	ND	6.3	ND	1.1 ± 2.6
	7.2°C	ND	4.5	3.6	ND	16.0	ND	4.0 ± 6.2
	12.8°C	173.5	176.9	158.0	423.6	764.0	214.7	318.4 ± 239.6
	Temp. Ramp I	23.5	ND	ND	ND	4.7	2.9	5.2 ± 9.2
	Temp. Ramp II	115.3	4.0	7.5	3.9	ND	27.6	26.4 ± 44.7

[1] ND = not detected
[2] Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
[3] Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

content of all mahi-mahi stored at 7.2°C and Temp. Ramp I and II for 5 days did not exceed 50 ppm.

The levels of putrescine, cadaverine, and histamine increased as decomposition progressed (Figure 27). Putrescine was not detected at initial decomposition but increased rapidly at advanced decomposition. Cadaverine was detected in small amounts initially but the quantity increased as decomposition progressed. Histamine was not detected in fresh muscle. Its formation did not occur until the stage of initial decomposition (7.2°C for 3 days). However, it increased dramatically after storage at a higher temperature (12.8°C) for 5 days. The formation of putrescine and cadaverine in mahi-mahi stored at 1.7°, 7.2°C, and Temp. Ramp I was faster than that of histamine. The levels of putrescine and cadaverine at these three temperatures were also higher than that of histamine at each interval. In contrast, the level of histamine was higher than those of putrescine and cadaverine in mahi-mahi stored at 12.8°C on day 5 of storage.

At the time of rejection (grade C), the contents of histamine in mahi-mahi stored at 7.2°, 12.8°C, and Temp. Ramp I and II were 4.0, 3.2, 5.2, and 26.4 ppm, respectively (Table 7). The trend for putrescine and cadaverine was the same as histamine. By the time of rejection, 7.8, 5.9, 7.0, and 15.6 ppm putrescine were formed for samples stored at 7.2°, 12.8°C, and Temp. Ramp I and II, respectively, whereas 8.2, 7.3, 9.8, and 29.6 ppm cadaverine were formed at the four respective temperatures. By the time of rejection, the sum of these three biogenic amines (Put+Cad+His) surpassed 20 ppm in mahi-mahi stored at 7.2°C and Temp. Ramp I and II. However, the combined content of these three amines was 16.4 ppm in samples stored at 12.8°C for 3 days. The sum of (Put+Cad+His) did not

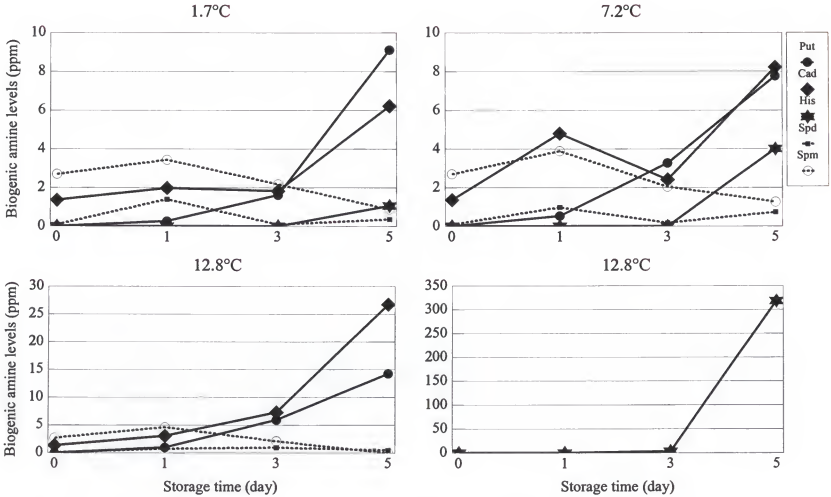
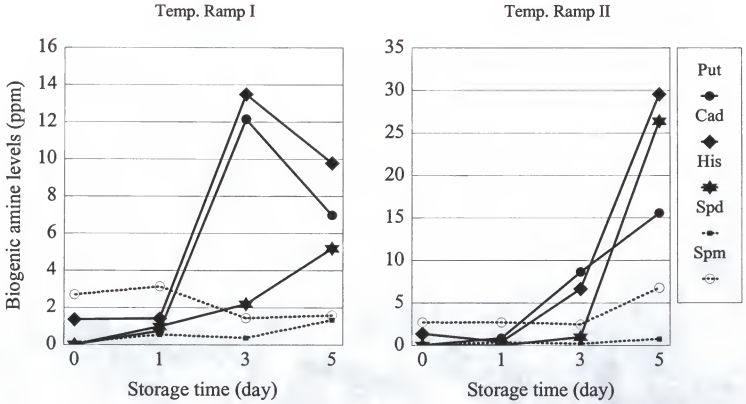


Figure 27. Time-related changes in biogenic amine content for mahi-mahi stored at different temperatures



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 27. (continued)

Table 7. GC analysis of biogenic amines (ppm) in mahi-mahi stored at different temperatures for up to 5 days

Day	Temperature	Put[1]	Cad	His	Spd	Spm	Put+Cad+His	Grade[2]
0	1.7°C	0.0 ± 0.0	1.4 ± 2.8	0.0 ± 0.0	0.1 ± 0.2	2.7 ± 1.0	1.4 ± 2.8	A
1	1.7°C	0.3 ± 0.4	2.0 ± 2.8	0.0 ± 0.0	1.4 ± 1.0	3.4 ± 1.6	2.2 ± 3.1	A
	7.2°C	0.5 ± 0.7	4.8 ± 4.0	0.0 ± 0.0	1.0 ± 1.0	3.9 ± 0.4	5.3 ± 4.2	A
	12.8°C	1.0 ± 1.0	3.1 ± 3.9	0.0 ± 0.0	0.7 ± 0.5	4.6 ± 2.7	4.1± 4.3	A
	Temp. Ramp I[3]	0.7 ± 1.2	1.4 ± 1.8	1.0 ± 2.4	0.6 ± 0.4	3.1 ± 1.9	3.1 ± 3.1	A
	Temp. Ramp II[4]	0.8 ± 0.7	0.4 ± 0.6	0.0 ± 0.0	0.4 ± 0.4	2.7 ± 0.3	1.3 ± 1.2	A
3	1.7°C	1.6 ± 1.1	1.8 ± 2.1	0.0 ± 0.0	0.1 ± 0.2	2.2 ± 0.6	3.5 ± 2.9	A
	7.2°C	3.3 ± 3.6	2.4 ± 1.2	0.0 ± 0.0	0.2 ± 0.3	2.0 ± 1.8	5.7 ± 4.6	B
	12.8°C	5.9 ± 8.7	7.3 ± 10.5	3.2 ± 3.6	1.0 ± 0.9	2.1 ± 1.3	16.4 ± 17.5	C
	Temp. Ramp I	12.2 ± 17.2	13.5 ± 10.0	2.2 ± 5.4	0.4 ± 0.5	1.4 ± 0.9	27.8 ± 27.0	B
	Temp. Ramp II	8.6±12.4	6.7 ± 3.1	1.0 ± 2.4	0.2 ± 0.3	2.5 ± 1.7	16.3 ± 14.7	B
5	1.7°C	9.1 ± 8.2	6.2 ± 2.8	1.1 ± 2.6	0.4 ± 0.4	0.9 ± 0.8	16.4±10.6	B
	7.2°C	7.8 ± 5.1	8.2 ± 5.2	4.0 ± 6.2	0.7 ± 0.5	1.3 ± 1.1	20.0 ± 12.9	C
	12.8°C	14.2±14.1	26.7 ± 28.5	318.4±239.6	0.5 ± 1.2	0.0 ± 0.0	359.4 ± 249.4	C
	Temp. Ramp I	7.0 ± 4.6	9.8 ± 4.7	5.2 ± 9.2	1.3 ± 1.1	1.6 ± 0.7	22.0 ± 13.9	C
	Temp. Ramp II	15.6 ± 8.5	29.6 ± 27.7	26.4 ± 44.7	0.8 ± 1.0	6.8 ± 5.1	71.5 ± 51.2	C

[1] Mean ± Standard deviation (n = 6)
[2] A: sensory rating 1-4 (fresh); B: sensory rating 4-6 (initial decomposition); C: sensory rating 6-10 (advanced decomposition, rejected)
[3] Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
[4] Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

exceed 20 ppm in mahi-mahi during 5 days of storage at 1.7°C. Therefore, the sum of (Put+Cad+His) can be used as an indicator of mahi-mahi spoilage.

Spermidine and spermine were present in smaller amounts in mahi-mahi. These two polyamines are usually present in fresh fish because they are necessary for cellular growth (Pollack et al., 1992). The level of spermine decreased slightly as decomposition progressed, while the level of spermidine remained the same or decreased slightly (Figure 27). Therefore, mahi-mahi decomposition had little influence on spermidine and spermine levels. This finding is in agreement with that of Kim and Bjeldanes (1979). They reported that the spermidine levels decreased slightly as tuna decomposed, whereas spermine levels remained the same for both good and decomposed tuna. Klausen and Lund (1986) reported that spermidine remained constant in herring (2-4 ppm) and mackerel (4-6 pp) stored at 2° and 10°C.

Veciana-Nogués et al. (1997) reported that spermidine and spermine were the prevailing biogenic amines found in fresh tuna samples at zero time. Putrescine and cadaverine were also found but at very low levels (< 0.5 ppm), while histamine was not detected. Mietz and Karmas (1978) also reported that the contents of histamine, putrescine, and cadaverine increased along with an apparent decease in spermidine and spermine, as decomposition of rockfish and salmon progressed.

In general, the levels of cadaverine found in decomposed mahi-mahi were higher than that of putrescine (Figure 27). Dainty et al. (1986) evaluated the bacterial sources of putrescine and cadaverine in chill-stored vacuum-packaged beef and concluded that cadaverine, which is probably formed through the decarboxylation of lysine, does not require

any metabolic input from other organisms. On the other hand, putrescine formation requires the growth of arginine-utilizing strains of lactic acid bacteria that are presumed to produce ornithine, which is subsequently decarboxylated by the Enterobacteriaceae. In addition, putrescine levels in spoiled fish are usually much lower than the levels of cadaverine, probably because of the limited quantities of ornithine in fish tissues. Putrescine levels in spoiled fish are usually less than 10 mg per 100 g (Kim and Bjeldanes, 1979; Staruszkiewicz and Bond, 1981). This may explain why the higher cadaverine levels relative to putrescine are usually found in decomposed samples.

Very good correlation ($r^2 = 0.9921$) was found between the CE and GC methods for detection of histamine in mahi-mahi fillets stored at 1.7°, 7.2°, and 12.8°C for 0, 1, 3, and 5 days (Figure 28). Good correlation means $r^2 \geq 0.9$. Histamine content of the same fish samples determined by GC were a little bit lower than those by CE. Histamine in methanolic extracts of mahi-mahi can be analyzed directly by CE without clean-up and derivatization. This rapid and sensitive method has a potential for on site screening of seafood and quantitation of histamine. The GC procedure for simultaneous analysis of 5 putrefactive amines provides a useful tool for assessing these chemical indicators in spoiled seafoods.

Good correlation was found between the sum of (Put+Cad+His) and bacterial count (Figure 29). The bacterial counts for mahi-mahi fillets were higher than 7 $\log_{10}$ CFU/g when more than 20 ppm (Put+Cad+His) were found. This result indicated that the microbial counts played an important role in the spoilage of seafood. High levels of (Put+Cad+His) were found in mahi-mahi fillets only when decomposition had occurred.

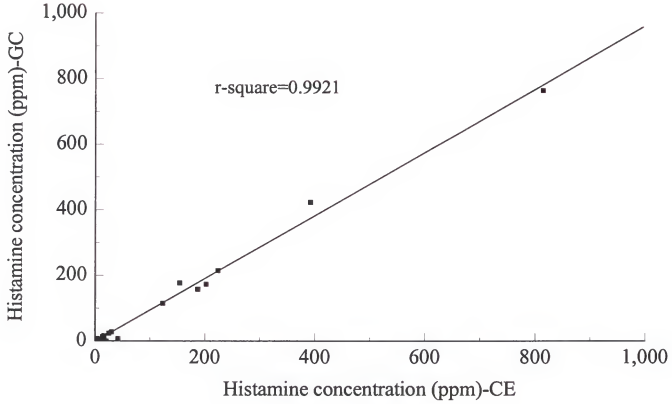


Figure 28. Correlation of CE and GC methods for histamine analysis in mahi-mahi stored at different temperatures for up to 5 days

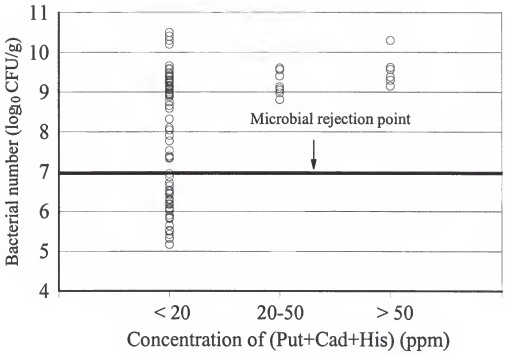


Figure 29. Correlation of bacterial numbers with biogenic amine concentrations in mahi-mahi stored at different temperatures for up to 5 days

Histamine Analysis Using Fluorometric Method

All calibration curves comparing the relationship between fluorescence intensity and histamine concentrations of standard solutions from 11 different trials were linear over the histamine range of 0-150 ppm (data not shown). When the data from these 11 trials were compiled and analyzed, a linear relationship of Y (fluorescence intensity) = 82.21X (concentration) - 0.045, with a correlation coefficient (r^2_{Ave}) ≥ 0.999, was obtained. The mean recovery rate of histamine from spiked mahi-mahi was found to be 92.1% over the tested concentrations of 50 - 500 ppm (Table 8). The average coefficient of variation (CV) for the intra-assay of histamine standard solutions was 1.7 and 1.0% for interassay. The average coefficient of variation (CV) for the intra-assay of histamine spiked mahi-mahi extracts was 1.6 and 3.1% for interassay. Thus, very good reproducibility was achieved with the fluorometric method for histamine analysis in both the standard solutions and fish extracts.

The accuracy and precision of the fluorometric method are comparable to those of the CE and GC methods. Two extracts of decomposed mahi-mahi sample were analyzed by the CE, GC, and fluorometric methods. The concentrations of histamine found in the first extract were 123.3, 115.3, and 112.6 ppm by CE, GC, and fluorometry analysis, and for the second extract were 202.4, 173.5, and 165.5 ppm, respectively.

Sensory Evaluation of Mahi-mahi Fillets

Sensory evaluation by the panel showed that some of the mahi-mahi fillets stored at 12.8°C started to release moderate fish odor after 1 day (Table 9). Some of the fillets stored at 7.2°C started to exhibit moderate discoloration and excessive odor after 3 days. Fish

Table 8. Recovery of histamine from fortified mahi-mahi by AOAC fluorometric method

Fortification level (ppm)	Conc. found (ppm)	Average recovery rate (% mean ± S.D., n=4)	Coefficient of variation (%)
50	49.4	98.8 ± 1.8	1.8
200	183.8	91.9 ± 1.8	2.0
500	428.1	85.6 ± 1.1	1.3
Mean		92.1	

Table 9. Time-related changes in sensory rating by a sensory panel for mahi-mahi fillets stored at different temperatures

Day	Temperature	Parameters				Mean ± S.D.
		Appearance	Color	Texture	Odor	
0		1.5[1]	1.5	1.5	1.5	1.5 ± 0.0
1	1.7°C	1.8	2.5	2.0	2.0	2.1 ± 0.3
	7.2°C	2.7	2.5	2.0	2.0	2.3 ± 0.3
	12.8°C	3.3	3.5	2.0	3.7	3.1 ± 0.8
	Temp. Ramp I[2]	2.5	2.8	2.0	2.0	2.3 ± 0.4
	Temp. Ramp II[3]	2.4	2.8	2.0	2.0	2.3 ± 0.4
3	1.7°C	2.8	4.0	3.5	3.0	3.3 ± 0.5
	7.2°C	4.8	5.3	5.8	6.0	5.5 ± 0.5
	12.8°C	7.8	5.8	7.0	7.9	7.1 ± 0.9
	Temp. Ramp I	5.5	5.5	3.5	3.5	4.5 ± 1.2
	Temp. Ramp II	4.8	5.8	3.5	3.5	4.4 ± 1.1
5	1.7°C	5.7	4.4	3.7	3.7	4.4 ± 0.9
	7.2°C	7.2	5.8	5.3	7.7	6.5 ± 1.1
	Temp. Ramp I	7.7	6.0	5.3	7.8	6.7 ± 1.2
	Temp. Ramp II	7.5	5.7	5.5	7.5	6.5 ± 1.1

[1] Each rating represents the mean of 10 judgements.
[2] Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
[3] Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

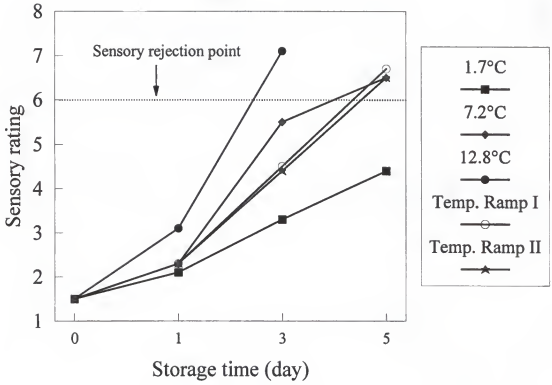
fillets stored at 1.7°C exhibited only slight fish odor and moderate discoloration after 5 days of storage. Both groups of mahi-mahi fillets that were stored at 1.7°C but exposed to 12.8°C for 24 hr showed moderate discoloration similar to that of 7.2°C after 3 days.

The mahi-mahi fillets stored at 12.8°C were deemed unacceptable (sensory rating > 6) after 3 days (Figure 30). Both groups of mahi-mahi fillets that underwent temperature ramp (Temp. Ramp I and II) and the mahi-mahi fillets stored at 7.2°C were considered unacceptable after 5 days. The mahi-mahi fillets stored at 1.7°C remained acceptable even after 5 days.

Most of the mahi-mahi fillets were rejected by the sensory panel when higher than 50 ppm (Put+Cad+His) were found (Figure 31). Most of the fillets were classified as grade B or C products when more than 20 ppm (Put+Cad+His) was detected. This indicated that decomposition of fish had occurred and higher levels of biogenic amines were found. Sensory analysis was not a good indicator of the presence of high levels of biogenic amines in fish. Monitoring of biogenic amine content in high risk fish species remains important for assurance of seafood quality and safety. Biogenic amines may be used as indicators for mahi-mahi quality and safety. López-Sabater et al. (1996) suggested that a lack of a relationship between histamine content and sensory attributes can explain the high incidence for scombrotoxicity.

AromaScan Analysis of Mahi-mahi Fillets

Results from AromaScan analysis of mahi-mahi stored at 1.7°C, 7.2°C, and 12.8°C for 3 time intervals (1, 3, and 5 days) are shown in Figure 32. The mappings of the five temperature groups were separated from that of day 0 samples in a time-related fashion.



Temp. Ramp. I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 30. Sensory rating of mahi-mahi fillets stored at different temperatures for up to 5 days

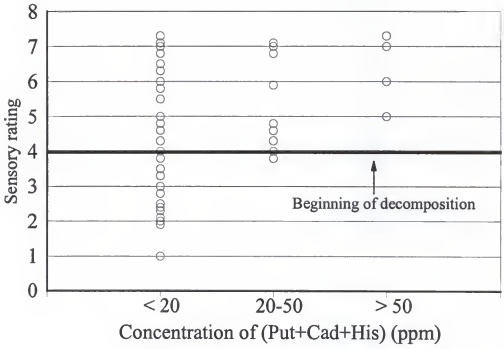
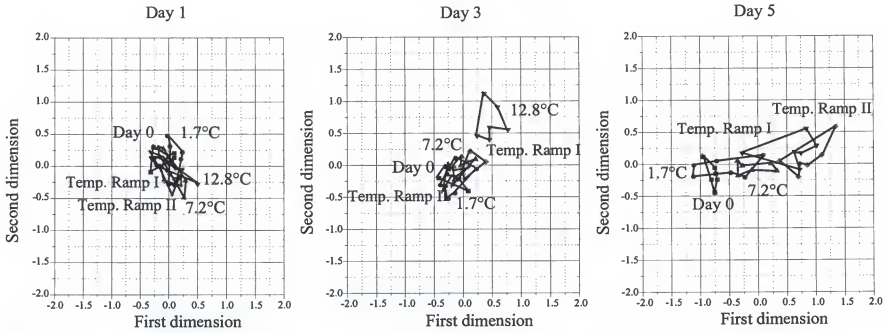


Figure 31. Correlation of sensory rating with biogenic amine content in mahi-mahi stored at different temperatures for up to 5 days



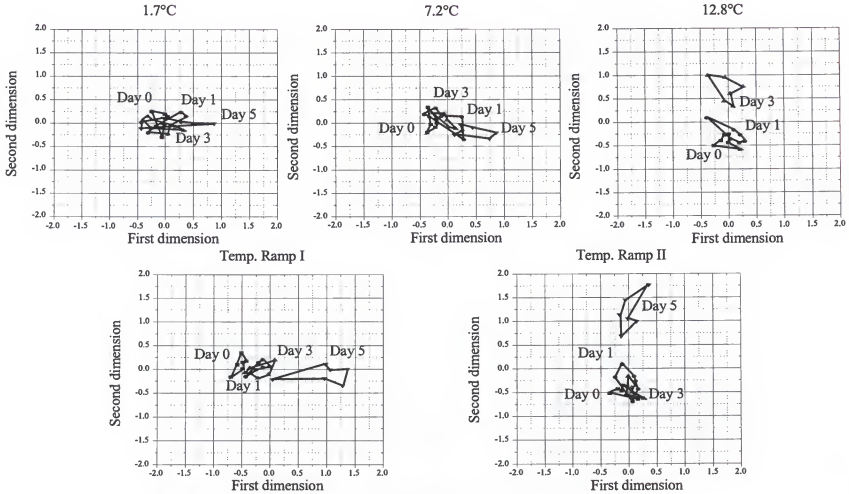
Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 32. Comparison of AromaMaps for mahi-mahi fillets stored at different temperatures on day 1, 3, and 5 of storage. All fillets were compared to day 0 controls.

Their maps continued to separate further as storage time increased. Mappings of the five temperature groups did not separate from each other on day1, indicating that the odor profiles for all fish fillets were similar on day 1. This result showed good correlation with sensory analysis which classified all the fillets from the five temperature groups as grade A fillets on day 1. Samples stored at 12.8°C for 3 days (grade C products by sensory analysis) started to show mappings that were separated from those of the other temperature groups and day 0 controls (grade A or B). Mappings of fillets stored at 7.2°C and Temp. Ramp I and II (grade C) started to separate from those of day 0 controls by day 5. Comparing the results from AromaScan and the sensory analysis, we found that AromaScan can identify odor differences between grade C fillets and those of grade A and B fillets.

AromaScan could not differentiate odor differences of mahi-mahi fillets stored at 1.7°C by days (Figure 33). Sensory analysis of mahi-mahi indicated that fillets stored at 1.7°C were grade A products during the 5 days of storage. Mappings of fillets stored at 7.2°C started to separate from day 0 controls on day 5 when fillets were rated as grade C products; whereas, fillets stored at 12.8°C started to separate from day 0 controls on day 3, when fillets were rated as grade C products. Similar results were also found in the two temperature ramp groups. Mappings of these two groups separated from day 0 controls when they became grade C products. These results indicated that AromaScan can differentiate the odor difference between grade C and grade A products.

Multiple discriminant analysis was used to show significant differences between samples. In discriminant analysis, the X and Y functions account for differences between different samples. The results from the multiple discriminant analysis showed that odor



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.

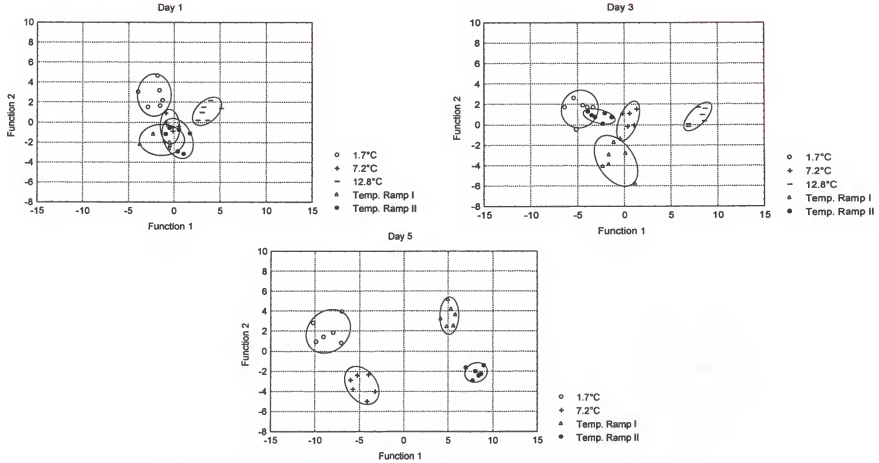
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 33. Comparison of AromaMaps for mahi-mahi fillets stored at 1.7°, 7.2°, 12.8°C, and temperature ramp I and II for various time periods. All fillets were compared to day 0 controls.

profiles from mahi-mahi stored at 7.2°C and those from the two temperature ramp groups were similar on day1. Their clusters did not separate from each other on the canonical discriminant graphs (Figure 34). The cluster of fillets stored at 1.7°C was well separated from those fillets stored at 12.8°C on day 1. This result is in agreement with the sensory analysis results which gave the fillets stored at 7.2°C and those from the two temperature ramp groups the same rating (2.3). Canonical discriminant graphs showed that mahi-mahi samples stored at different temperatures were separated from each other in a time-related fashion. Their clusters continued to separate further as the storage time increased. Clusters of samples stored at closer temperatures showed less spatial separation on the graphs. The different stages of decomposition shown in mahi-mahi stored at different temperatures were clearly grouped into distinct regions of the plot on day 5. The separation of the clusters generally correlated with the sensory rating.

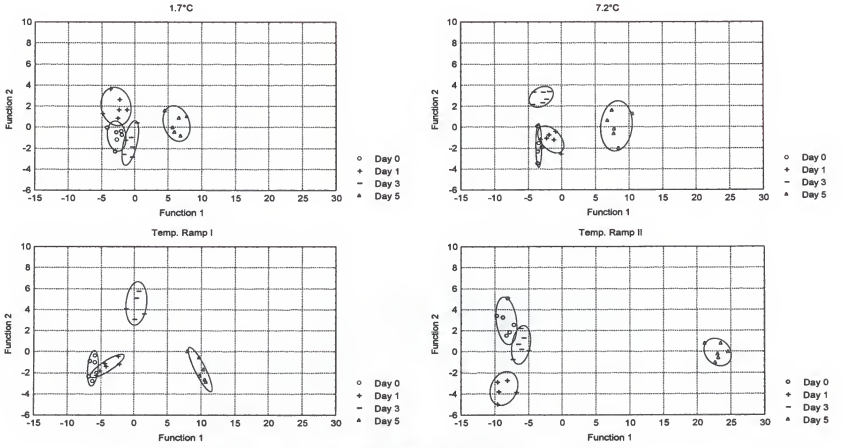
Canonical discriminant graphs (Figure 35) showed that clusters of day 0, 1, and 3 samples stored at1.7°C did not completely separate from each other. However, the cluster for day 5 samples separated from those of day 0, 1, and 3 clusters. Clusters of mahi-mahi samples showed a temperature-related separation in the plots. As the temperature increased, the clusters separated further in the canonical discriminant graphs. These results indicated that AromaScan is capable of identifying the changes of odor profiles in mahi-mahi fillets during storage.

Clusters of mahi-mahi fillets stored at different temperatures with different microbial loads separated from each other in the canonical discriminant graphs (Figure 36). Clusters with close bacterial numbers were also closer to each other on the graphs. A high correlation



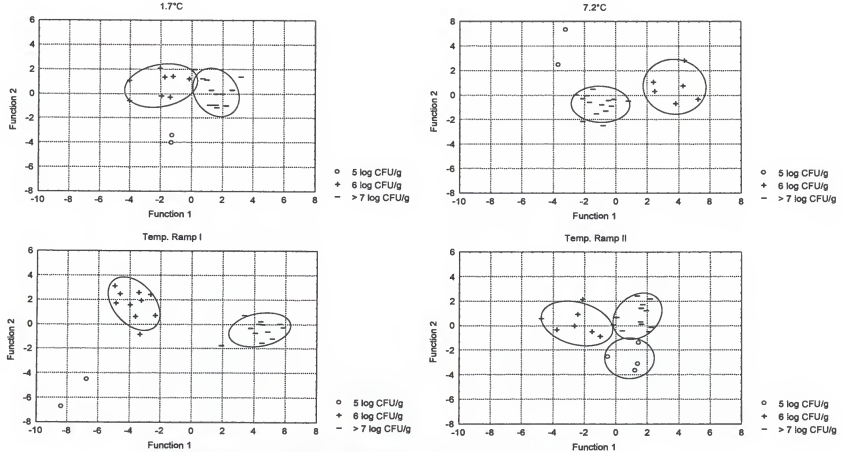
Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 34. Canonical discriminant graphs for mahi-mahi fillets stored at different temperatures on day 1, 3, and 5 of storage



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 35. Canonical discriminant graphs for mahi-mahi fillets stored at 1.7°, 7.2°C, and temperature ramp I and II for various time periods



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 36. Correlation of AromaScan analyses with bacterial counts in mahi-mahi fillets stored at different temperatures

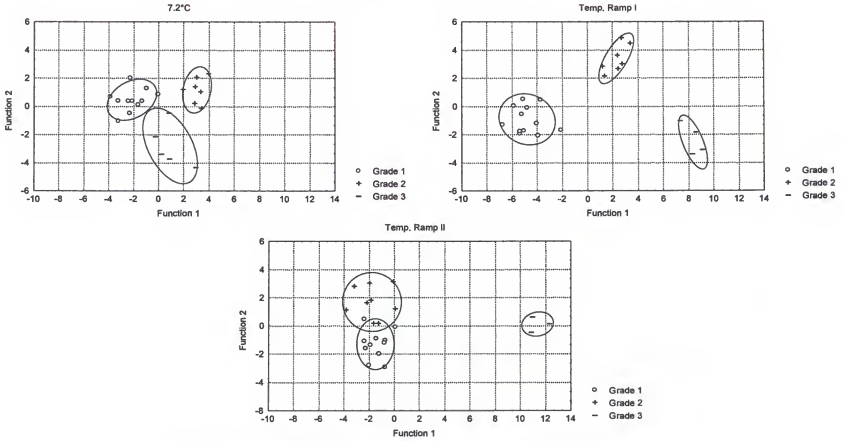
was also found between the AromaScan and sensory analysis. AromaScan can be used to predict the grade of mahi-mahi fillets stored at 7.2°C and Temp. Ramp I and II (Figure 37). The cluster of grade C fillets was farther away from those of grade A and B in mahi-mahi stored at the two temperature ramp groups. This indicated that the grade C fillets had an odor profile far different from those of grades A and B fillets in both temperatures. Figure 38 shows that AromaScan can be used to predict the content of biogenic amines in mahi-mahi stored under Temp. Ramp II condition. The three levels of (Put+Cad+His) indicated whether the fillet was fresh (< 20 ppm), under initial decomposition (20-50 ppm), or under advanced decomposition (> 50 ppm). This result indicated that AromaScan is capable of detecting differences in odor profiles in mahi-mahi under different stages of decomposition, and that it can be used for quality and freshness evaluation.

The results of this study demonstrate a correlation between AromaScan and sensory evaluation conducted by a panel. Comparison of the AromaScan results with microbiological measurements also shows a similar effect. Thus, loss of freshness in mahi-mahi can be objectively determined by AromaScan analysis.

Tuna Fillets Stored at 0°, 4°, 10°, and 22°C

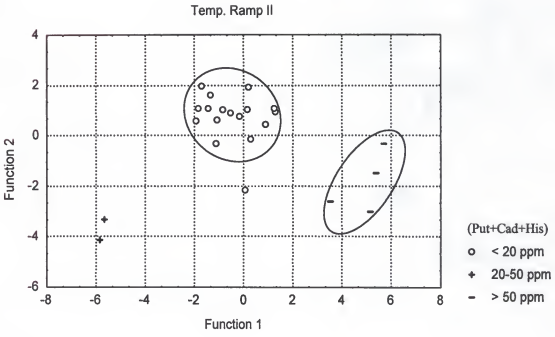
Total Microbial Counts

Bacteria grew rapidly on tuna samples stored at 10° and 22°C (Figure 39). By day 3, total aerobic counts on PCA reached a level of 7-8 $\log_{10}$ CFU/g tuna. This value was not reached in tuna samples stored at 0° and 4°C until after 9 days. It was noted that, as histamine level exceeded 50 ppm in tuna fillets stored at all four temperatures, the total



Temp. Ramp I: 1.7°C for 24 hours, then 12.8°C for 24 hours before returning to 1.7°C.
Temp. Ramp II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 37. Correlation of AromaScan analyses with sensory grading for mahi-mahi fillets stored at different temperatures



Temp. Ramp. II: 1.7°C for 48 hours, then 12.8°C for 24 hours before returning to 1.7°C.

Figure 38. Correlation of AromaScan analyses with biogenic amine content in mahi-mahi fillets stored at temperature ramp II

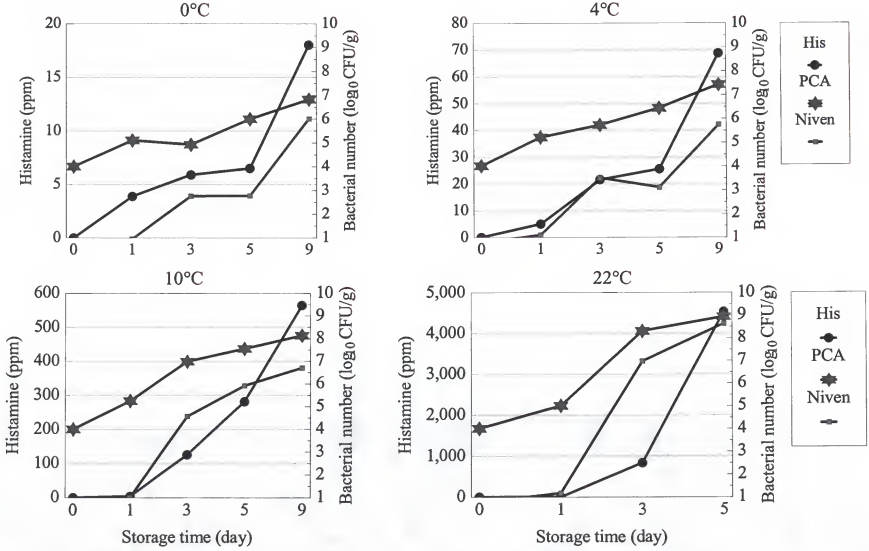


Figure 39. Correlation of histamine formation with growth of total bacteria and histamine-producing bacteria

bacterial counts reached 7 $\log_{10}$ CFU/g or higher. These cut-off levels agree with the generally accepted microbiological standards for fresh fish. Thus, the presence of high numbers of bacteria is a good indicator of a possible histamine hazard. Compliance with the microbiological criteria should assure safety from scombroid poisoning. However, to serve as a practical screening test for fish safety, a more rapid test than enumeration by total aerobic counts is needed.

Isolation and Identification of Histamine-Producing Bacteria

The number of histamine-forming bacteria was very low at the beginning of the test (Figure 39). This was confirmed by the negligible histamine content initially present. However, during the subsequent storage, the counts for histamine-forming bacteria increased rapidly, especially for those stored at 22°C. Growth of histamine-producing bacteria showed good agreement with the formation of histamine in tuna. A high level of histamine (> 50 ppm) was found in tuna when histamine-producing bacteria reached 4 $\log_{10}$ CFU/g or higher. It should be noted that histamine formation occurred earlier than the detection of histamine-forming bacteria in samples stored at 0° and 4°C. Storage on ice and refrigeration at 4°C did not prevent the growth of microorganisms containing histidine decarboxylase activity.

Histamine-producing bacteria were found in fillets stored at all four different temperatures. Except for the 22°C fillets, the counts of histamine-forming bacteria were similar for fillets stored at 0°, 4°, and 10°C. The higher storage temperature (22°C) provided better growth conditions for histamine-producers and histamine production. A total of 6 bacterial species were isolated and identified from the decomposing fish. *Morganella morganii* were mainly isolated from fillets stored at 22°C. *Enterobacter agglomerans*,

Enterobacter intermedium, *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Serratia liquefaciens* were the other histamine-forming bacteria found in tuna fillets. These results showed a good agreement with those reported by López-Sabater et al. (1996). They isolated *Morganella morganii, Enterobacter agglomerans*, *Enterobacter intermedium*, *Pseudomonas fluorescens*, and *Serratia liquefaciens* as histamine-forming bacteria from tuna fish (*Thunnus thynnus*) decomposed at 0°, 8°, and 20°C. They also isolated *Morganella morganii* and *Enterobacter agglomerans* from sections stored at 20°C. In contrast, *Serratia liquefaciens* and *Enterobacter intermedium* were more prevalent in samples stored at 8°C. The *Morganella morganii* they isolated showed a very active histidine decarboxylase activity (> 300 mg histamine/100 g tuna after 18 hr incubation at 37°C), while *Enterobacter agglomerans* was able to form more than 100 mg histamine/100 g tuna. The remaining isolates were all identified as weak histamine formers. Only those organisms able to produce more than 100 mg histamine/100 g fish during a short incubation would be of concern as a potential toxicological hazard.

Numerous bacteria have been reported to possess histidine decarboxylase activity (Taylor et al., 1978). *Klebsiella pneumoniae*, *Morganella morganii*, and *Hafnia alvei* were the most common organisms implicated in the formation of toxicologically significant levels of histamine in fish (Stratton and Taylor, 1991). Frank et al. (1981) reported that the Enterobacteriaceae were the organisms primarily responsible for decomposition of scombroid fish and these had the ability to decarboxylate histidine to histamine. Taylor et al. (1978) reported that *Morganella morganii* was the most prolific histamine producer in trypticase soy broth histidine (TSBH) medium. It had the capability of forming sufficient

histamine in scombroid fish products to elicit food poisoning outbreaks. They also demonstrated that *Serratia liquefaciens* had the capability to produce histamine in TSBH medium.

Morganella morganii, *Proteus vulgaris*, and *Pseudomonas fluorescens* have been isolated from Spanish mackerel and all showed histidine decarboxylase activity (Middlebrooks et al. 1988). *Morganella morganii* had been reported to possess ornithine decarboxylase, while *Serratia liquefaciens* was shown to possess both lysine and ornithine decarboxylase (Taylor and Sumner, 1986).

Capillary Electrophoresis Determination of Histamine

CE electropherograms for histamine standard, blank muscle extracts, histamine-spiked samples, and tested tuna fillets did not appear to have interference with the histamine peak (Figure 40). The migration time for pure histamine was 3.2 min at constant voltage of 10 kV using a 24 cm x 50 μm capillary cartridge. A very high linear relationship (r^2_{Ave} > 0.999) occurred with peak areas and concentrations over 0.5 to 100 ppm histamine standard solutions. A 100.6% recovery rate was achieved from histamine-spiked tuna fillets over the concentration range of 25 to 100 ppm.

Tuna is one of the most common fish species involved in scombroid fish poisoning, a representative of scombroid family of fish. Muscle of scombroid fish contain large amounts of histidine which can be converted to histamine, forming toxic levels. The scombrotoxin outbreak occurring in South Carolina in 1989 was caused by eating yellowfin tuna with high levels of histamine (5830 and 7280 ppm) and resulted in 9 cases of foodborne diseases (MMWR, 1989).

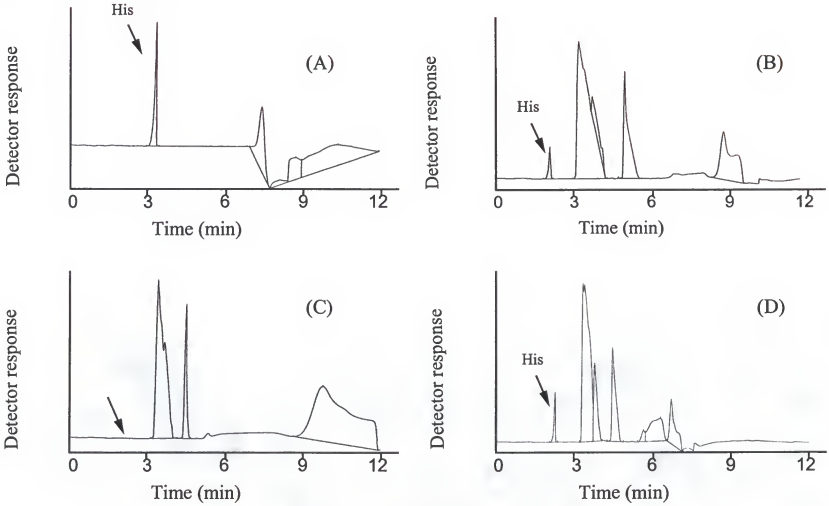


Figure 40. Typical electrophoretograms of (A) histamine standard solution, (B) muscle extracts of histamine-spiked tuna, (C) blank tuna, and (D) treated tuna fillets

Histamine content of yellowfin tuna was negligible at the start of storage. Frank et al. (1981) also reported that fresh tuna contains negligible amounts of histamine immediately after catching, but the level of histamine increases with the progression of fish decomposition. Formation of histamine occurred very quickly at 22°C, reaching toxic levels (> 50 mg/100 g; FDA, 1982) in three days (Table 10). The increase in histamine was delayed at refrigerated temperatures (4° and 10°C) but notable amounts were detected after 3 days at 10°C and 5 days at 4°C, exceeding the limit of 50 ppm (FDA, 1996). The level of histamine in tuna stored at 0°C did not exceed the 50 ppm tolerance level even after 9 days. These results were similar to those reported by Silva et al. (1998) on the formation of histamine in big-eye and skipjack tuna stored at 4°, 10°, and 22°C. The results indicated that temperature plays an important role in histamine formation, a low refrigeration temperature is critical in reducing histamine formation.

A high variation in histamine content was found among tuna fillets stored at the same temperature for the same period of time (Table 10). Arnold and Brown (1978) reported that histamine levels varied greatly depending on the location of the fillet in the fish. Histamine in tunafish showed an uneven distribution, with the highest content in the nape (anterior section) and the lowest in the tail (posterior section). Both the uneven distribution of histamine in fish flesh and differences in sensitivity between individuals have been used to explain why some people became ill after eating tuna with a high level of histamine while others who had eaten the same meal failed to show symptoms. Hence, it has been concluded that the assay of histamine content would not serve as a good index of tuna quality (López-Sabater et al., 1996).

Table 10. Histamine concentrations (ppm) in yellowfin tuna stored at different temperatures for up to 9 days as analyzed by CE method

Day	Temperature	Fillet sample					Mean ± S.D.
		1	2	3	4	5	
0		ND[1]	ND	ND	ND	ND	0.0 ± 0.0
1	0°C	10.2	11.9	ND	9.1	ND	6.2 ± 5.8
	4°C	12.7	ND	12.5	10.4	15.4	10.2 ± 6.0
	10°C	ND	18.3	ND	ND	15.9	6.8 ± 9.4
	22°C	ND	ND	ND	ND	ND	0.0 ± 0.0
3	0°C	18.8	ND	ND	7.6	12.4	7.8 ± 8.1
	4°C	ND	25.4	43.2	33.6	8.5	22.1 ± 17.8
	10°C	135.6	117.5	122.7	102.6	208.6	137.4 ± 41.5
	22°C	1022.0	584.3	933.2	980.6	1502.5	1004.5 ± 328.1
5	0°C	ND	ND	18.9	21.4	ND	8.1 ± 11.1
	4°C	71.7	29.2	24.7	12.9	12.3	30.1 ± 24.3
	10°C	62.3	51.3	528.9	342.5	283.4	253.7 ± 201.3
	22°C	4769.7	4175.4	5124.3	4800.2	5256.0	4825.1 ± 418.6
9	0°C	29.2	10.0	12.8	28.6	19.0	19.9 ± 8.9
	4°C	51.7	252.5	14.8	19.1	120.0	91.6 ± 99.3
	10°C	1318.7	153.3	699.1	422.0	491.4	616.9 ± 438.2

[1] ND = not detected

Histamine Analysis Using Fluorometric Method

A 93.8% recovery rate was achieved from histamine-spiked tuna fillets over the concentration range of 25 to 100 ppm using AOAC fluorometric method. The results for the AOAC method of histamine levels in tuna samples were showed in Table 11. A very good correlation ($r^2 = 0.9972$) was found between AOAC and CE methods for determination of histamine in tested tuna samples (Figure 41). The AOAC method is sensitive and reproducible (Stratton and Taylor, 1991) but is complicated and time consuming. It required cleanup steps to remove amino acids that interfere with the pH-sensitive derivatization step with o-phthalicdicarboxyaldehyde (OPT). The OPT derivative is relatively unstable and the fluorescence intensity diminishes rapidly (Staruszkiewicaz et al., 1977). CE method was rapid, simple, sensitive, and specific. It may offer additional application as a rapid and confirmatory alternative procedure for seafood speciation. It is suitable to serve as a screening method for routine analysis.

Gas Chromatography Determination of Biogenic Amines

Our study clearly indicated that decomposition of mahi-mahi had little influence on spermidine and spermine contents. Therefore, only the contents of putrescine, cadaverine, and histamine were determined in treated tuna samples. The GC conditions used in the mahi-mahi study were slightly modified to better resolve the three amine peaks in tuna extracts.

Very good correlation was found between the GC and CE methods ($r^2 = 0.9952$) as well as between the GC and AOAC fluorometric methods ($r^2 = 0.9961$) for histamine determination in tuna samples (Figure 41). Therefore, the CE method can be used for quick

Table 11. Histamine concentrations (ppm) in yellowfin tuna stored at different temperatures for up to 9 days as analyzed by AOAC fluorometric method

Day	Temperature	Steak sample					Mean ± S.D.
		1	2	3	4	5	
0		2.8	1.7	1.7	1.7	2.3	2.0 ± 0.5
1	0°C	8.5	11.1	2.8	6.0	1.7	6.0 ± 3.9
	4°C	10.2	0.5	9.0	5.4	10.8	7.2 ± 4.3
	10°C	0.5	21.9	0.5	ND[1]	11.4	6.9 ± 9.7
	22°C	2.8	2.8	2.8	1.9	2.5	2.5 ± 0.4
3	0°C	22.5	3.3	2.8	6.0	13.2	9.6 ± 8.4
	4°C	3.2	30.0	50.8	30.8	5.4	24.0 ± 19.9
	10°C	152.9	143.9	156.7	120.9	202.7	155.4 ± 29.9
	22°C	988.4	573.3	935.7	1014.8	1573.8	1017.2 ± 358.6
5	0°C	0.1	0.1	21.5	20.5	ND	8.4 ± 11.5
	4°C	72.0	32.3	25.7	12.3	10.9	30.6 ± 24.8
	10°C	61.1	52.0	628.6	402.9	285.7	286.1 ± 243.1
	22°C	5073.0	4315.4	4988.8	5409.7	5662.2	5089.8 ± 509.9
9	0°C	32.8	9.5	12.3	33.3	21.6	21.9 ± 11.1
	4°C	59.8	235.1	15.9	27.1	111.6	89.9 ± 89.3
	10°C	1471.3	178.4	727.1	482.5	564.2	684.7 ± 482.8

[1] ND = not detected

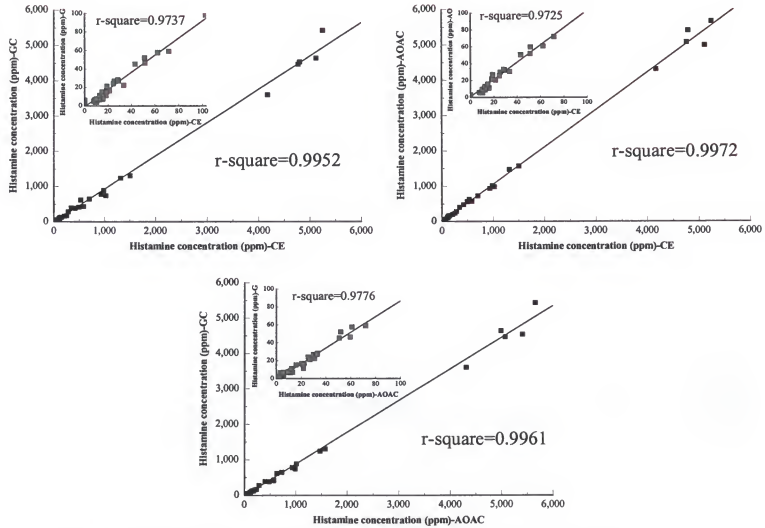


Figure 41. Correlation of CE, AOAC, and GC methods for analysis of histamine in tuna

screening of fish samples for histamine hazard. Samples found to have 20 ppm histamine by CE method can then be confirmed using the AOAC fluorometric or GC method.

Gas chromatography of PFP derivatives for amine standards and muscle extracts of blank, amine-spiked, and tested tuna fillets did not appear to have interference with amine peaks (Figure 42). The recovery rates for amines were 104.5, 107.9, and 77.3% for putrescine, cadaverine, and histamine, respectively.

Great changes in the content of putrescine, cadaverine, and histamine were observed throughout the storage of tuna at the four temperatures (Table 12), especially at 10° and 22°C. Histamine was the prevailing biogenic amine found in the test samples, followed by cadaverine. A great increase in cadaverine and a slight increase in putrescine were also observed. A similar order of biogenic amine formation was observed by Veciana-Nogués et al. (1997) in tuna stored at 0°, 8°, and 20°C. Histamine was also the prevailing biogenic amine found during spoilage of sardine (Ababouch et al., 1991). However, as shown in our study for mahi-mahi stored at 1.7°, 7.2°, and 12.8°C (reported in the previous section) and other fish species (Fernández-Salguero and Mackie, 1987a; Middlebrook et al., 1988), the formation of putrescine or cadaverine during spoilage was similar or greater than that of histamine.

Polyamines were absent in fresh (day 0) tuna sample (Table 12). Less than 20 ppm of histamine was found in tuna stored at 0°C for 9 days. Putrescine was not formed at 0°C during 9 days of storage. Negligible amounts of cadaverine were found in tuna during 9 days of storage at 0°C. Putrescine and cadaverine were not found in tuna stored at 4°C for 3 days although they were rejected by the sensory panel. These fillets had only 21.6 ppm of

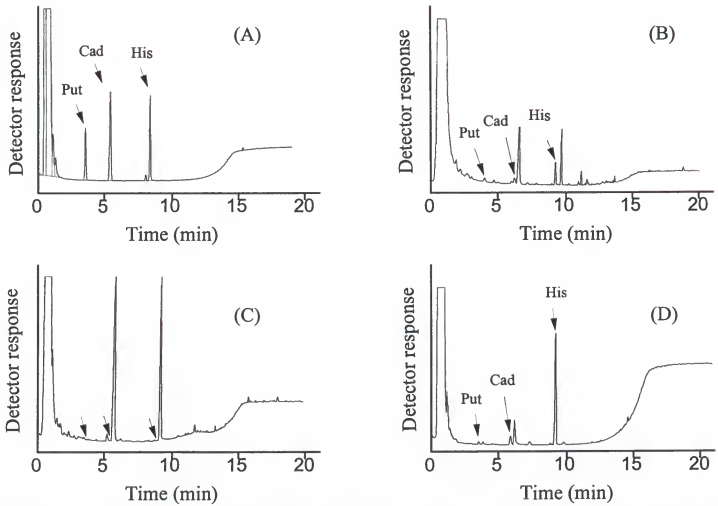


Figure 42. Gas chromatograms of PFP derivatives of (A) a mixture of standard biogenic amines, (B) muscle extracts of biogenic amines-spiked tuna, (C) blank tuna, and (D) treated tuna fillets

Table 12. GC analysis of biogenic amines (ppm) in tuna stored at different temperatures for up to 9 days

Day	Temperature	Put[1]	Cad	His	Put+Cad+His	Grade[2]
0		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	A
1	0°C	0.0 ± 0.0	0.1 ± 0.2	3.9 ± 3.0	4.0 ± 3.0	A
	4°C	0.0 ± 0.0	0.1 ± 0.2	5.0 ± 3.1	5.1 ± 3.1	A
	10°C	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 5.6	4.0 ± 5.6	A
	22°C	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 1.3	0.6 ± 1.3	A
3	0°C	0.0 ± 0.0	0.0 ± 0.0	5.9 ± 7.0	5.9 ± 7.0	A
	4°C	0.0 ± 0.0	0.0 ± 0.0	21.6 ± 16.1	21.6 ± 16.1	C
	10°C	0.0 ± 0.0	2.2 ± 2.6	125.4 ± 23.2	127.7 ± 21.4	B
	22°C	35.8 ± 13.3	146.7 ± 43.9	832.0 ± 315.9	1014.5 ± 354.0	A
5	0°C	0.0 ± 0.0	0.0 ± 0.0	6.5 ± 8.9	6.5 ± 8.9	A
	4°C	1.7 ± 3.8	0.0 ± 0.0	25.6 ± 20.4	27.3 ± 23.9	B
	10°C	17.1 ± 10.3	15.1 ± 17.5	281.0 ± 238.8	314.9 ± 262.1	C
	22°C	36.1 ± 12.5	164.7 ± 68.7	4533.0 ± 648.6	4733.9 ± 614.8	C
9	0°C	0.0 ± 0.0	0.0 ± 0.0	18.0 ± 9.2	18.0 ± 9.2	B
	4°C	9.2 ± 6.9	12.5 ± 11.9	68.8 ± 64.1	90.5 ± 76.0	C
	10°C	23.9 ± 12.9	44.9 ± 40.4	563.9 ± 421.4	632.7 ± 473.4	C

[1] Mean ± Standard deviation (n = 5)

[2] A: sensory rating 1-4 (fresh); B: sensory rating 4-6 (initial decomposition); C: sensory rating 6-10 (advanced decomposition, rejected)

histamine. A higher content of histamine (281 ppm) was found in tuna stored at 10°C at the time of rejection, whereas only 17 and 15 ppm putrescine and cadaverine, respectively, were formed. Greater amounts of putrescine (36 ppm), cadaverine (165 ppm), and histamine (4533 ppm) were found in tuna stored at 22°C at the time of rejection. At the time of rejection, the sum of (Put+Cad+His) exceeded 20 ppm no matter whether the fillets were stored at 4°, 10°, or 22°C. However, not all the fillets were rejected by the sensory panelists when the sum of (Put+Cad+His) exceeded 20 ppm. This result indicated that organoleptic analysis is not adequate to prevent histamine intoxication in tuna.

Good correlation was found between the sum of (Put+Cad+His) and total bacterial counts in tuna fillets stored at different temperatures (Figure 43). Bacterial counts for tuna fillets were less than 7 $\log_{10}$ CFU/g when (Put+Cad+His) was less than 20 ppm. Whereas, the total bacterial counts reached 7 $\log_{10}$ CFU/g or higher when most of the fillets had more than 20 ppm (Put+Cad+His). It is noted that histamine-producing bacteria reached 3 $\log_{10}$ CFU/g or higher in tuna when more than 20 ppm (Put+Cad+His) were found. These results indicated that bacteria played an important role in the formation of biogenic amines in tuna. Middlebrook et al. (1988) reported that putrescine and cadaverine are biogenic amines commonly related to fish spoilage. Neither histamine-producing bacteria nor biogenic amines were found on day 0 of storage. Thus, these results indicated that the tuna was under advanced decomposition or spoilage when more than 20 ppm (Put+Cad+His) were found. The sum of (Put+Cad+His) can be used as an indicator of tuna spoilage and histamine hazard.

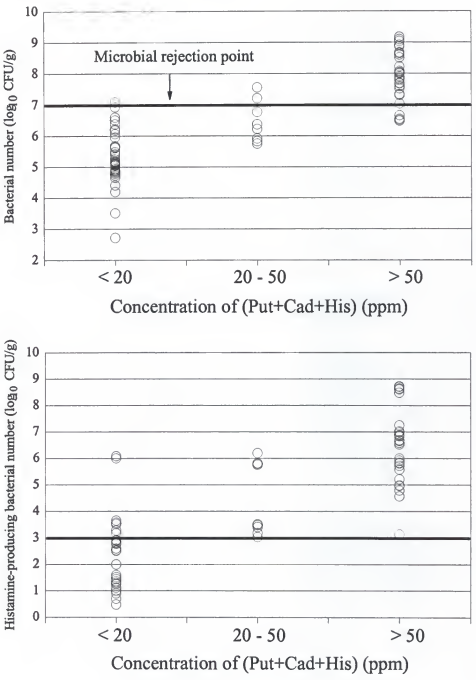


Figure 43. Correlation of bacterial numbers with biogenic amine content in tuna

Veciana-Nogués et al. (1997) reported that putrescine and cadaverine were found at very low levels (< 0.5 ppm) in fresh tuna samples at zero time when histamine was not detected. Rogers and Staruszkiewicz (1997) also reported that very fresh tuna contained < 0.1 ppm cadaverine and putrescine. Commercial grade products frequently contain 0.2 - 0.3 ppm amines. Organoleptic classification of the decomposed product generally correlates with levels of either cadaverine or putrescine above 0.5 ppm (Rogers and Staruszkiewicz, 1997). No free histamine was found in fresh tuna; while the presence of histamine indicates decomposition (Rogers and Staruszkiewicz, 1997). Our results confirmed that when tuna was classified as grade A fillet, the levels of putrescine and cadaverine were < 0.1 ppm (Table 12). When tuna was under initial decomposition (grade B product), the level of cadaverine was 2.2 ppm in tuna stored at 10°C for 3 days and the level of putrescine was 1.7 ppm in tuna stored at 4°C for 5 days. Therefore, putrescine and cadaverine can be used as indicators for tuna decomposition.

It was obvious that higher storage temperatures accelerated the formation of putrescine, cadaverine, and histamine. Histamine content reached 125 ppm and 832 ppm in 3 days of storage at 10° and 22°C, respectively, although the muscles were still considered acceptable organoleptically (Table 12). Frank et al. (1985) reported that the formation of biogenic amines correlated more with the activity of mesophilic than psychrotrophic bacteria, which could explain the more extensive formation of those amines at 22°C.

The presence of biogenic amines can also be useful in estimating the freshness or degree of spoilage of fish because these compounds are found at very low levels in fresh fish, and their formation is associated with bacterial spoilage (Fernández-Salguero and Mackie,

1987a). The FDA recommended the use of other scientific data to judge fish freshness, such as the presence of other biogenic amines associated with fish decomposition (FDA, 1996). Furthermore, biogenic amines can be used as indicators of poor-quality raw material in preserved fish products because they are thermally stable compounds (Sim et al., 1992).

Histamine is the most common amine formed during decomposition of red meat fishes. During decomposition of mahi-mahi and tuna in this study, the predominant amine formed was histamine. However, there was a remarkable difference in histamine formation between the two species. In the case of tuna stored at 10°C, the histamine level exceeded 50 ppm at day 3 even at acceptable stage. For mahi-mahi, histamine level did not exceed 50 ppm until after 5 days of storage at 12.8°C when they became unacceptable. Levels of putrescine, cadaverine, and histamine formed in tuna after 5 days of storage at 10°C were similar to those formed in mahi-mahi stored at 12.8°C.

The amount of each amine (putrescine, cadaverine, or histamine) formed during spoilage depends on the type of bacterial contamination and the particular condition of decomposition to which the product is subjected to. In general, high levels of histamine and cadaverine occurred in scombrotoxic fish (such as tuna and mahi-mahi) due to the occurrence of spoilage before chilling. Elevated histamine and putrescine levels may be due to decomposition caused by unsanitary practices in food service operations (Rogers and Staruszkiewicz, 1997).

Sensory Evaluation of Tuna Fillets

Fresh raw tuna fillets had a firm texture with typical red color and no off-odor. The color and odors of test fillets changed with increased storage time and temperature. Fillets

stored at the four temperatures showed similar physical appearance at day 1 and were considered to have the same quality as fresh tuna (Table 13). Those fillets stored at 0°C exhibited only slight fish odor and slight discoloration after 3 days of storage. Samples stored at 4° and 10°C started to produce putrefactive odors and showed undesirable color change and surface slime by day 3. Fillets stored at 22°C showed only slight fish odor and a nice red color similar to fresh tuna fillets by day 3; they were scored as the best fillets at day 3 by sensory panelists. Fillets stored at 10° and 22°C produced strong putrefactive odors by day 5 and were rejected by panelists. Those fillets stored at 4°C were rated unacceptable by day 9, while tuna fillets were still considered acceptable even after 9 days when stored at 0°C. The increase in sensory scores followed that of total bacterial growth (Figure 39 and Figure 44). Sensory analysis of tuna samples generally agreed with total bacteria counts at rejection point. When tuna was classified as grade C fillets, the total bacterial counts of most fillets reached 7 $\log_{10}$ CFU/g or higher (Figure 45). The histamine-producing bacterial numbers reached 3 $\log_{10}$ CFU/g or higher in all grade B and C fillets. Thus, bacteria played an important role in tuna spoilage.

Myoglobins and cartenoids account for the red color of fresh tuna. Meat tissue that contains primarily myoglobin (deoxymyoglobin) is purplish-red in color. Binding with the molecular oxygen yields oxymyoglobin, and the color of the tissue changes to the customary bright red. Oxidation of myoglobin and oxymyoglobin yields the undesirable brownish-red color of metmyoglobin. Such oxidation of myoglobin and oxymyoglobin occurs more slowly at higher pH values. Furthermore, the rate of autoxidation of oxymyoglobin occurs more slowly than that of myoglobin (Von Elbe and Schwartz, 1996). In our study, the color of

Table 13. Time-related changes in sensory rating by a sensory panel for tuna fillets stored at different temperatures for up to 9 days

Day	Temperature	Defects				Mean ± S.D.
		Appearance	Discoloration	Texture	Odor	
0		1.3[1]	1.3	1.3	1.2	1.3 ± 0.1
1	0°C	1.5	1.5	1.9	1.6	1.6 ± 0.2
	4°C	1.8	1.6	1.7	2.0	1.8 ± 0.1
	10°C	2.0	1.8	1.9	1.9	1.9 ± 0.1
	22°C	1.8	1.6	2.0	2.0	1.9 ± 0.2
3	0°C	3.9	3.5	3.8	3.7	3.7 ± 0.2
	4°C	6.5	7.1	5.9	6.3	6.5 ± 0.5
	10°C	5.2	5.2	5.1	7.0	5.6 ± 0.9
	22°C	3.1	3.1	3.4	2.7	3.1 ± 0.3
5	0°C	3.6	3.2	4.1	3.1	3.5 ± 0.5
	4°C	6.0	6.5	5.8	5.5	5.9 ± 0.4
	10°C	7.1	8.1	6.1	7.2	7.1 ± 0.8
	22°C	5.8	5.4	6.3	9.8	6.8 ± 2.0
9	0°C	5.0	6.0	5.5	5.4	5.5 ± 0.4
	4°C	6.9	7.9	6.6	7.2	7.2 ± 0.6
	10°C	6.2	6.6	6.1	9.1	7.0 ± 1.4

[1] Each rating represents the mean of 11 judgements.

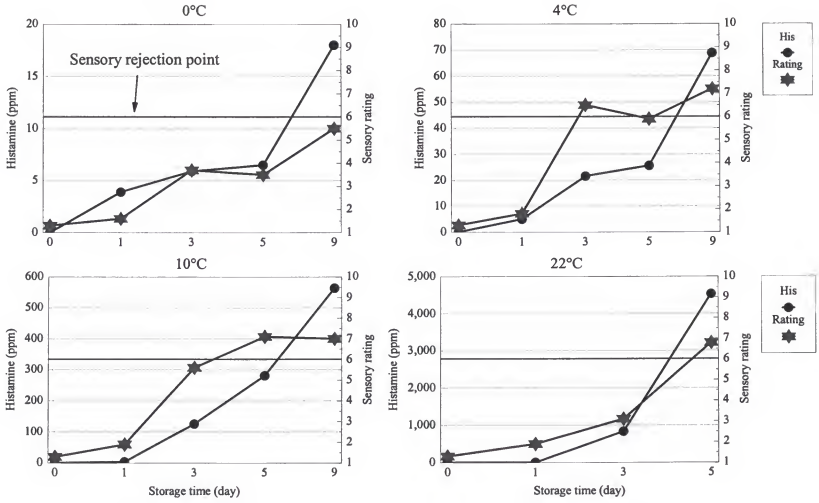


Figure 44. Correlation of histamine formation with sensory analysis in tuna fillets stored at different temperatures

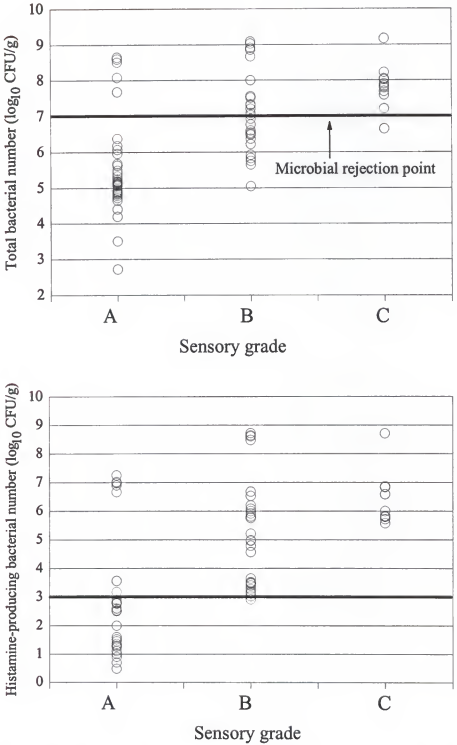


Figure 45. Correlation of sensory grading with bacterial counts in tuna fillets

tuna stored at 22°C did not change much during storage. The maintenance of tuna red color is probably due to pH changes in tuna during storage, thus, slowing down the oxidation of myoglobin and oxymyoglobin. High levels of biogenic amines were found in tuna after 3 days of storage at 22°C. The formation of biogenic amines increased the pH of tuna muscles which helps maintain the red color.

Tuna fillets stored at 0°C for 9 days had a histamine content of less than 20 ppm (Figure 39), even though the level of histamine-producing bacteria was 6 $\log_{10}$ CFU/g. This result suggests that low temperature storage cannot prevent the growth of histamine-producing bacteria, but may inhibit the histidine decarboxylase activity. This finding is in agreement with the results of López-Sabater et al. (1996) who reported that even though a high count (10^5 cells per g) of *M. morganii* or *K. oxytoca* (probably the two most powerful histamine-formers known) were present in tuna, no histamine would be formed by them, if a proper refrigeration (0°C) was applied. Hence, storage of fish at 0°C or lower temperatures would be the best way to control histamine formation in fish.

At the time of rejection (sensory rating > 6) the histamine content in tuna stored at 4°C was less than 50 ppm (Figure 44). Higher levels of histamine were formed only after deterioration of tuna which made it unsuitable for human consumption. By the time of rejection, the total bacterial counts in tuna exceeded 7 $\log_{10}$ CFU/g. These results suggest that, when tuna is properly stored at low non-freezing temperatures, histamine formation would not represent a serious health risk to consumers unless the tuna was mishandled previously. Thus, chilling below 4°C is effective in preventing histamine formation in tuna.

Decomposition of fish, followed by histamine formation in samples stored at 10°C, was faster than at 0° and 4°C (Figure 44). Histamine formation remained negligible during the first 24 hr, but began to increase rapidly, reaching concentrations higher than the FDA limit by day 3. The organoleptic rejection point was reached on day 5 and the tuna was considered unpleasant and inedible. Therefore, before decomposition was evident by sensory criteria, enough histamine had accumulated to cause a health problem. For example, at the sensory evaluation score of 6 (limit of rejection) or higher, the histamine content in these fillets had increased to 564 ppm (130 to 1242 ppm). Therefore, consumption of tuna stored at 10°C for extended periods can be hazardous to consumers. López-Sabater et al. (1996) reported that although tuna stored at 8°C only developed minor external signs of decomposition, the histamine content in the muscle reached 1410 to 1690 ppm.

Histamine formation occurred very rapidly in tuna maintained at 22°C (Figure 45). Histamine formation was negligible during the first 24 hr of storage, but after initial appearance of this biogenic amine, the rate of increase was extremely fast. Before decomposition was evident by sensory analysis, enough histamine (434 to 1308 ppm) had accumulated to cause a health problem (Table 14). By the time of rejection (sensory score > 6), histamine content had increased to 4533 ppm (3604 to 5430 ppm). These results suggest that tuna exposed to high temperature abuse would represent a serious health risk to consumers due to histamine formation, and physical appearance is not a good criterion to evaluate histamine-related health hazard. This could explain the high incidence of this type of food poisoning.

Table 14. GC analysis of histamine (ppm) in tuna stored at different temperatures for up to 9 days

Day	Temperature	Steak sample					Mean ± S.D.
		1	2	3	4	5	
0		ND[1]	ND	ND	ND	ND	0.0 ± 0.0
1	0°C	7.0	6.5	1.8	4.1	ND	3.9 ± 3.0
	4°C	6.9	ND	6.9	4.0	7.2	5.0 ± 3.1
	10°C	ND	11.4	ND	ND	8.8	4.0 ± 5.6
	22°C	ND	2.8	ND	ND	ND	0.6 ± 1.3
3	0°C	17.0	ND	ND	5.3	7.1	5.9 ± 7.0
	4°C	6.6	26.9	45.3	22.4	6.9	21.6 ± 16.1
	10°C	130.4	118.0	119.1	98.4	161.4	125.4 ± 23.2
	22°C	742.3	434.2	783.1	892.4	1308.2	832.0 ± 315.9
5	0°C	ND	ND	15.9	16.6	ND	6.5 ± 8.9
	4°C	58.9	26.8	24.2	9.8	8.2	25.6 ± 20.4
	10°C	57.6	52.0	617.6	392.9	284.8	281.0 ± 238.8
	22°C	4464.1	3603.8	4636.8	4530.9	5429.5	4533.0 ± 648.6
9	0°C	26.9	7.7	11.5	28.2	15.5	18.0 ± 9.2
	4°C	46.41	170.69	15.38	21.25	90.0	68.8 ± 64.1
	10°C	1242.0	129.7	648.9	384.8	414.0	563.9 ± 421.4

[1] ND = not detected

AromaScan Analysis of Tuna Fillets

AromaScan analysis of tuna stored at 0°, 4°C, 10°, and 22°C for 1, 3, 5, and 9 days are shown in Figure 46. Mappings of the fillets stored at these four temperatures were separated from the day 0 samples in a time-related fashion. Their maps continued to separate further as the storage time increased. Mappings for the four temperature groups were not separated from each other at day1, indicating similar odor profiles in these four groups. This result correlated well with the sensory analysis results which classified all the fillets from these four temperature groups as grade A product at day 1. However, the mappings of the day 3 fillets did not separate from each other although the sensory analysis showed differences in fillet quality. A good agreement was found between AromaScan and sensory analysis with fillets stored at the four temperatures by day 5. Fillets with similar sensory rating can not be separated from each other on the mappings. The larger difference in sensory score, the more separation occurred with the mappings.

Mappings for tuna fillets stored at 0°C were not separated from each other by days of storage (Figure 47). This indicated the quality of tuna stored at 0°C did not change much during storage. Tuna fillets stored at different temperatures showed a temperature-related separation. As the temperature increased, the trend of separation increased. Comparing the results from AromaScan and sensory analysis, it was found that AromaScan can identify odor differences between grade C fillets and those of grade A and B fillets. At 10°C, the mappings for day 5 and 9 fillets (sensory rating > 6, grade C) started to separate from those of day 0, 1, and 3 (sensory rating < 6, grade A and B). Thus, AromaScan can identify the degree of decomposition in tuna during storage. A good agreement was also found between

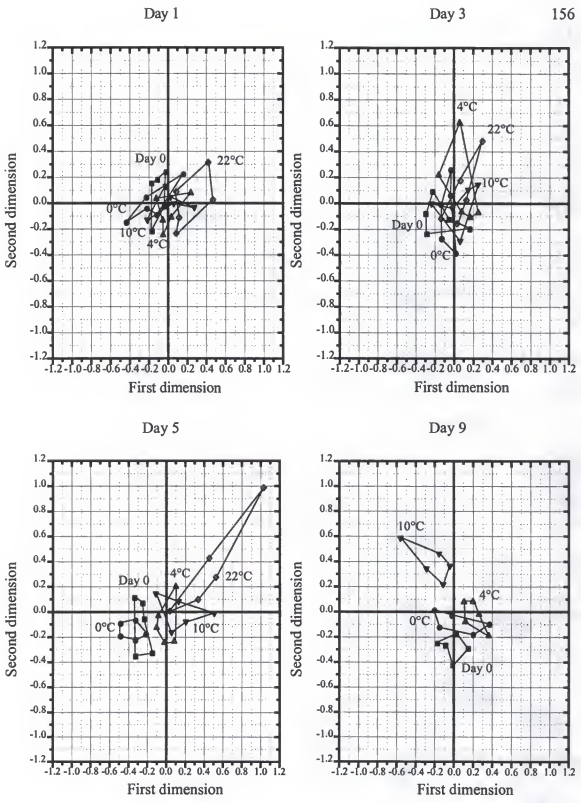


Figure 46. Comparison of AromaMaps for tuna fillets stored at different temperatures on day 1, 3, 5, and 9 of storage. All fillets were compared to day 0 controls.

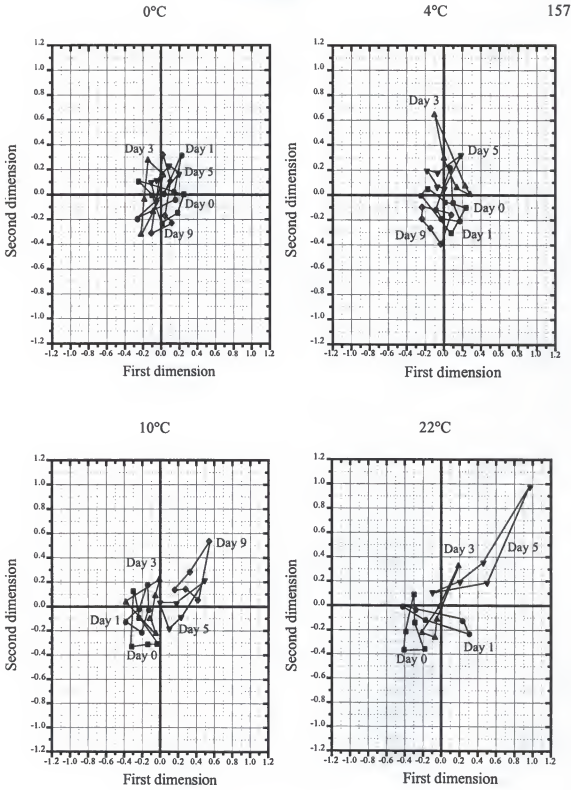


Figure 47. Comparison of AromaMaps for tuna fillets stored at 0°, 4°, 10° and 22°C for various time periods. All fillets were compared to day 0 controls.

AromaScan and sensory analysis of fillets stored at 22°C. Fillets with similar sensory rating do not separate from each other on mappings. The larger difference in sensory score, the more separation was found with the mappings.

Multiple discriminant analysis was used to show significant differences between samples by the spatial separation between the clusters. In discriminant analysis, the X and Y functions account for differences between different samples. The results showed that odor profiles from tuna stored at the four temperatures could be differentiated by days and grouped into separate clusters on canonical discriminant graphs (Figure 48).

Canonical discriminant graphs (Figure 49) showed that the clusters of tuna samples stored at the same temperature were closer to each other in the plots when the day of storage was closer. The clusters of tuna samples showed a temperature-related separation in the plots, the clusters of fillets stored at 22°C showed more spatial separation than those stored at 0°, 4°, and 10°C. The clusters of tuna stored at 4°C for 3, 5, and 9 days did not separate well from each other. This indicated that the AromaScan considered the quality of these fillets to be similar. This result is in good agreement with the sensory evaluation which classified those fillets as grade C products. A good agreement was also found between AromaScan and sensory analysis (odor rating) of fillets stored at 10°C. Canonical discriminant graphs showed that the clusters for day 0 and 1 fillets (with similar odor rating of 1.2 and 1.9) were close to each other, while the clusters for day 3 and 5 fillets (with similar odor rating of 7.0 and 7.2) were close to each other. However, the clusters of these fillets were further separated from that of day 9 fillets (odor rating of 9.1). This result indicated that AromaScan is capable of differentiating odor changes occurring during tuna storage.

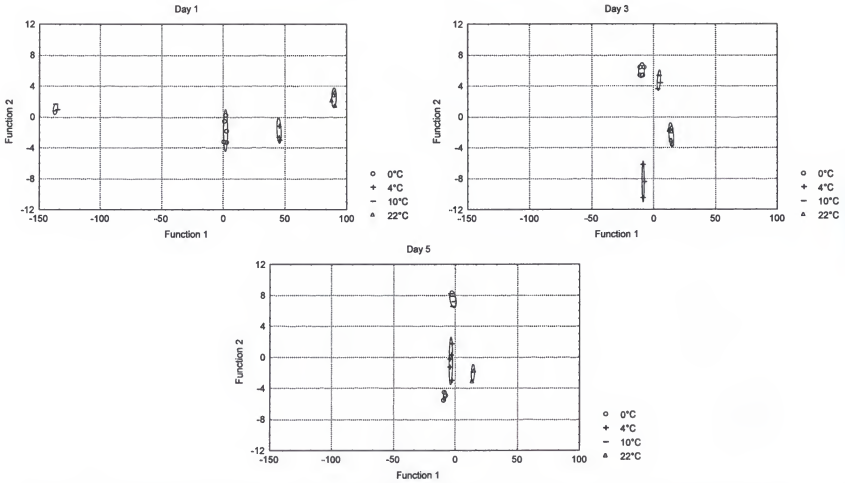


Figure 48. Canonical discriminant graphs for tuna fillets stored at different temperatures on day 1, 3, and 5 of storage

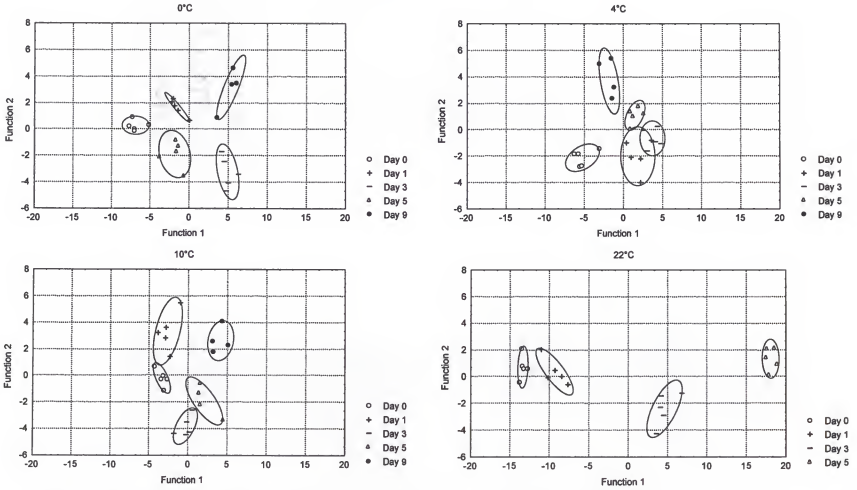


Figure 49. Canonical discriminant graphs for tuna fillets stored at 0°, 4°, 10°, and 22°C for various time periods

The AromaScan also predicted the grade of tuna fillets stored at 4°, 10°, and 22°C (Figure 50). A temperature-related relationship occurred with the cluster separation of sensory grade; the clusters of tuna fillets stored at 22°C were more separated than those at 4° or 10°C. Figure 51 showed that AromaScan can be used to predict the level of biogenic amines in tuna stored at 4°C. The clusters of fillets with different levels of histamine content were separated from each other in the graph. Fillets with the total biogenic amines (Put+Cad+His) less than 20 ppm can be separated from those having more than 20 ppm (Put+Cad+His) in the canonical discriminant graph. Thus, AromaScan can be used for quality and safety evaluation of tuna.

The clusters of tuna fillets stored at different temperatures with different microbial loads also separated from each other in the canonical discriminant graphs (Figure 52). They also showed a temperature-related separation in the plots; as the temperature increased, the clusters were separated more. The clusters of fillets with close total bacterial counts were close to each other on the graphs. The clusters of tuna fillets stored at different temperatures with different histamine-producing bacterial numbers separated from each other in the canonical discriminant graphs (Figure 53). However, the results from the canonical discrimant graph showed that the AromaScan analysis had a better correlation with histamine-producing bacterial number than with the total bacterial counts.

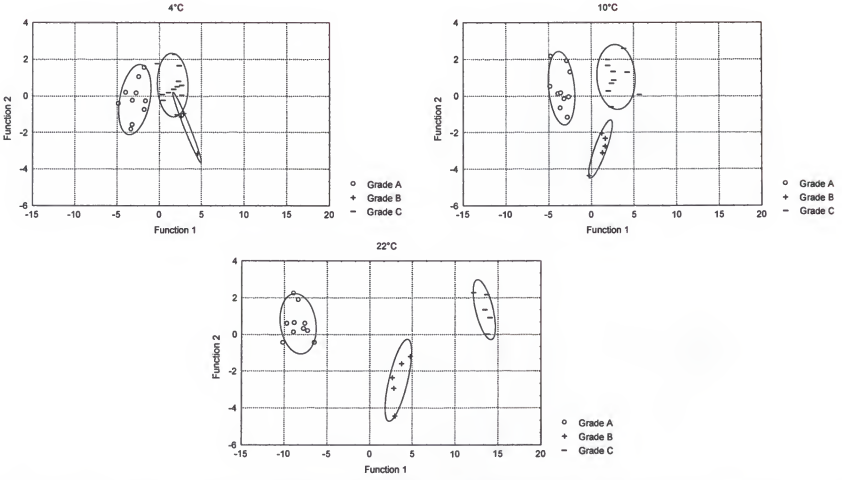


Figure 50. Correlation of AromaScan analyses with sensory grading for tuna fillets stored at different temperatures

Multiple Discriminant Analysis

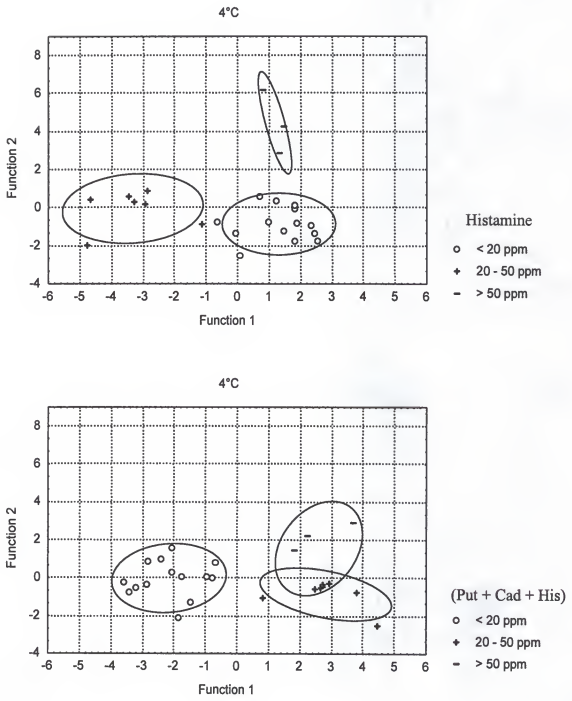


Figure 51. Correlation of AromaScan analyses with biogenic amine content in tuna fillets stored at 4°C

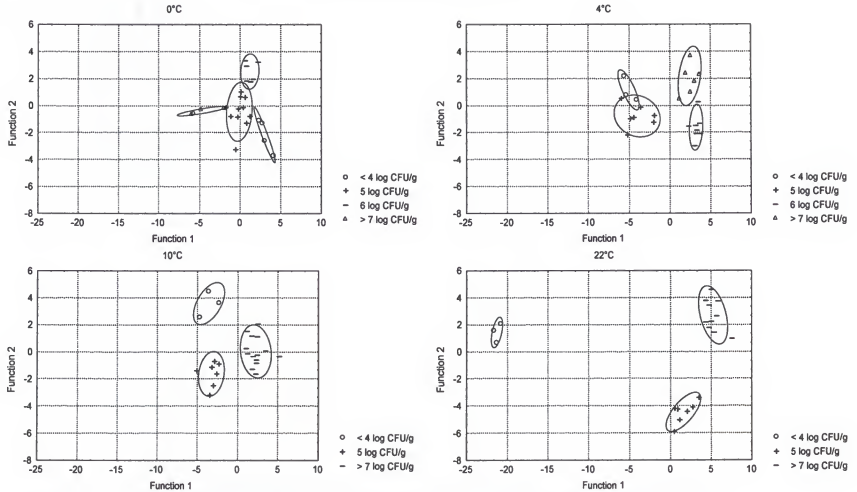


Figure 52. Correlation of AromaScan analyses with total bacterial counts in tuna fillets stored at different temperatures

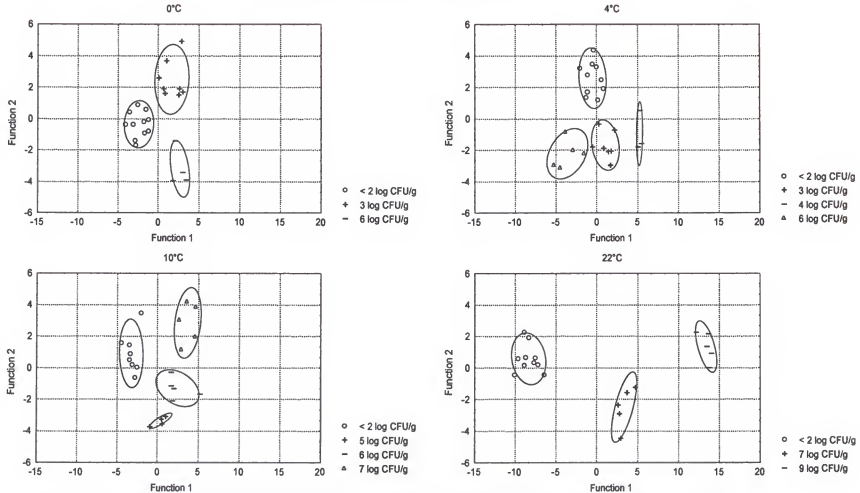


Figure 53. Correlation of AromaScan analyses with numbers of histamine-producers in tuna fillets stored at different temperatures

CHAPTER 5
SUMMARY AND RECOMMENDATIONS

Salmon fillets stored at -20°, 4°, and 10°C as well as those stored at 1.7°, 7.2°, and 12.8°C showed a time- and temperature-related increase in bacterial loads. *Pseudomonas putrefaciens* and *Moraxella* spp. were identified to be the bacterial species responsible for histamine production on positive salmon fillets. A 90.9% recovery rate was achieved from histamine-spiked salmon fillets over the concentration range of 25 to 250 ppm by CE. Histamine levels ranging from <0.5 to 54.7 ppm were found in salmon fillets. Histamine production in salmon fillets was not always associated with the sensory changes for smell/odor of the fish. A good correlation was found between sensory grade and bacterial count. The microbial counts indicated that bacteria played an important role in causing spoilage of fresh salmon fillets. Sensory evaluation is a good test for indicating fish quality. AromaScan can detect odor changes of salmon fillets during storage. A good correlation was found between AromaScan and microbial loads. AromaScan was able to predict the grade of salmon fillets stored at different temperatures.

Stored mahi-mahi fillets showed time- and temperature-related increases in bacterial loads. The change in fish quality correlated with increases in bacterial numbers. Bacterial levels were useful and objective indicators of gross spoilage for mahi-mahi. High levels of histamine (154 to 817 ppm) were detected in fish after 5 days of storage at 12.8°C. Less than

50 ppm histamine was found in mahi-mahi fillets stored at 1.7° and 7.2°C for 5 days. The levels of putrescine, cadaverine, and histamine increased as decomposition progressed, while the levels of spermidine and spermine decreased. Putrescine was not detected at initial decomposition but increased rapidly as decomposition advanced. Cadaverine was detected in small quantities at the initial decomposition stage but increased as decomposition progressed. Histamine was not detected in fresh muscle. Its formation did not occur until the initial stage of decomposition. The formation of putrescine and cadaverine in mahi-mahi stored at 1.7°, 7.2°C, and Temp. Ramp I was faster than that of histamine. The levels of putrescine and cadaverine at these three temperatures were also higher than that of histamine at each time interval. Fillets stored at 7.2° and 12.8°C were considered unacceptable after 5 and 3 days of storage, respectively. Fillets stored at 1.7°C were still considered acceptable even after 5 days of storage. A good correlation was found between the total biogenic amines (Put+Cad+His) and bacterial counts in mahi-mahi fillets. High levels of (Put+Cad+His) were found in mahi-mahi fillets only when decomposition of fillets had occurred. A good correlation was also found between the sensory rating and bacterial numbers of the mahi-mahi fillets during storage. Most of the mahi-mahi fillets were rejected by sensory analysis when (Put+Cad+His) was higher than 50 ppm. Sensory analysis was not a good indicator of high levels of biogenic amines in mahi-mahi.

Monitoring biogenic amines in high risk fish species remains important for assurance of seafood quality and safety. Biogenic amines may be used as indicators for mahi-mahi quality and safety. Human sensory study provides a useful means to monitor both changes in freshness and the onset of spoilage. AromaScan has the capacity to identify the changes

in odor profiles in mahi-mahi fillets during storage. The change in quality as detected by AromaScan correlated with that of microbiological measurements as well as with that of sensory analysis. The loss of freshness and decreased quality in mahi-mahi fillets can be objectively determined by AromaScan analysis.

Stored tuna fillets showed time- and temperature-related increases in bacterial loads. The change in tuna quality correlated with increases in bacterial numbers. Bacterial level is a useful and objective indicator of gross spoilage and the presence of a histamine hazard in tuna. Histamine producing bacteria were found in fillets stored at all four different temperatures. *Morganella morganii*, *Enterobacter agglomerans*, *Enterobacter intermedium*, *Pseudomonas fluorescens*, *Proteus vulgaris*, and *Serratia liquefaciens* were identified as the bacterial species responsible for histamine production in tuna fillets. The growth of histamine-producing bacteria showed good agreement with the formation of histamine in tuna.

The presence of bacteria and temperature were important for the formation of biogenic amines in tuna; a low refrigeration temperature during storage is critical for reducing histamine formation. When tuna has undergone high temperature abuse, histamine formation would represent a serious health risk to consumers. Physical appearance alone is not a good criterion to evaluate a histamine-related health hazard. Histamine was the prevailing biogenic amine during tuna storage at 0°, 4°, 10°, and 22°C. It was formed at the highest concentration followed by cadaverine. A greater increase in cadaverine and a slight increase in putrescine were observed during storage of tuna. A good correlation was found between the total biogenic amines (Put+Cad+His) and total bacterial counts in tuna fillets

stored at different temperatures. The (Put+Cad+His) can be used as an indicator of tuna spoilage and presence of histamine hazard. The change of quality as detected by AromaScan correlated with that of microbiological measurements. AromaScan can be used to grade the degree of decomposition in tuna during storage.

Overall, storage temperature and the microbial counts played important roles in the spoilage of fresh seafood. The monitoring of bacterial levels is a useful objective indicator of gross spoilage and the presence of histamine hazard in mahi-mahi and tuna. Histamine level has little value in monitoring the loss of freshness that occurs in fish fillets during storage at different temperatures. Biogenic amines may be used as quality and safety indicators for mahi-mahi and tuna. Very good correlations ($r^2 > 0.99$) were found among AOAC, CE, and GC methods for determination of histamine in tested mahi-mahi and tuna samples. The CE method may provide an alternative procedure for rapid screening and determination of the histamine content of seafood. The GC procedure for simultaneous analysis of 5 putrefactive amines provides a useful tool for assessing these chemical indicators in spoiled seafoods. A comparison of these three methods for determination of histamine in tuna is showed in Table A-8. Human sensory study provides a useful means to monitor both changes in freshness and the onset of spoilage. AromaScan analysis may provide a viable, quantitative approach for determining fish freshness which could be used for quality control and inspection purposes. It can do this objectively and in a short analysis time (12 minutes). It can also be used as a quality control instrument to assist a sensory panel in evaluating and controlling seafood products. The system's neural network can be trained with representative aromas/odors from the different classes of seafoods enabling a

rapid classification to be made either at source or at any point along the supply and distribution channels. Results were sufficient to encourage a more extensive and detailed study.

Further work should be done for determination of fish freshness using a sensory panel and AromaScan. Using a 10-point sensory scale for determination of fish grade should be studied further to more accurately classify fish to grade A, B, or C. A study should be conducted to compare the accuracy and correlation of sensory analytical methods using a 10-point scale and grade A, B, or C. More fish samples and species should be analyzed by AromaScan to collect sufficient data for training the artificial neural network (ANN). After appropriately trained with representative aromas/odors from the different classes of seafoods, the ANN can recognize odors in real time and be able to rapidly classify an unknown seafood sample. AromaScan can then be used in a HACCP plan for monitoring seafood freshness in the future.

APPENDIX

Table A-1. Biogenic amine (hydrochlorides) standard mixtures

Mixture	Biogenic amines	Put	Cad	His	Spd	Spm
1	Stock vol. (ml)	0.03	0.03	0.15	0.03	0.05
	Conc. (ppm)	3	3	15	3	5
2	Stock vol. (ml)	0.1	0.1	0.25	0.1	0.1
	Conc. (ppm)	10	10	25	10	10
3	Stock vol. (ml)	0.2	0.5	0.4	0.2	0.2
	Conc. (ppm)	20	50	40	20	20
4	Stock vol. (ml)	0.35	0.75	0.75	0.35	0.35
	Conc. (ppm)	35	75	75	35	35
5	Stock vol. (ml)	0.5	1	1	0.5	0.5
	Conc. (ppm)	50	100	100	50	50

Put: putrescine, Cad: cadaverine, His: histamine, Spd: spermidine, and Spm: spermine. The concentration of each amine stock solution is 1 mg/ml. The table indicates the volume of each amine stock solution needed for preparing 10 ml of each standard mixture at the specified concentration.

Table A-2. Sensory evaluation sheet for determination of the quality of salmon fillet

Fish fillet: Date: No.:

Defect	Degree	A1	A2	A3	A4
Discoloration	Slight				
	Moderate				
	Excessive				
Odor	Slight				
	Moderate				
	Excessive				

Defect	Degree	B1	B2	B3	B4
Discoloration	Slight				
	Moderate				
	Excessive				
Odor	Slight				
	Moderate				
	Excessive				

Defect	Degree	C1	C2	C3	C4
Discoloration	Slight				
	Moderate				
	Excessive				
Odor	Slight				
	Moderate				
	Excessive				

Table A-3. Score sheet for grading salmon fillet

Sample:		Treatment:										Experiment No.										Day:										Date:									
Fish No.		I										II										III																	
Panelist No.		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Appearance defects (flesh consistency)	Slight																																								
	Moderate																																								
	Excessive																																								
Discoloration	Slight																																								
	Moderate																																								
	Excessive																																								
Odor	Slight																																								
	Moderate																																								
	Excessive																																								
Total minor																																								
Total major																																								
Total serious																																								
Subsample grade																																								
Total grade A																																								
Total grade B																																								
Total substandard																																								
Total sample grade																																								
Remarks																																								

Table A-4. Sensory scale (10 points) for judging the quality attributes of fresh, raw salmon (*Salmo salar*) or mahi-mahi (dolphin fish, *Coryphaena hippurus*) fillets

General appearance

Rating scale:
1 - Attractive, fresh, clean, intact, full bodied fillet; excellent, preferred product
4 - Fair, acceptable appearance with some noticeable changes due to loss in full body, and initial color changes evidenced as beiging, some dehydration at edges, possible some initial slime.
6 - Fair, less appealing yet acceptable but not preferred. More evidence of beiging with initial banding of color along dorsal length and lower portions and belly. Possibly initial beige with slight oranging - brown tints. More slime evident and tacky surface emerging.
10 - Reject due to obvious slime, color banding and loss of appeal.

Meat color

Rating scale:
1 - Fresh salmon or mahi-mahi, no discoloration of meat or connective tissues, rich and attractive.
4 - Fair, acceptable, yet some initial changes evident as beiging and/or banding.
6 - Fair, less appealing more obvious loss of color, beiging, initial brown tints, yet acceptable by average standards.
10 - Reject - distinct objectionable color changes, loss of color, beige-brown to orange colors.

Gapping: Separation of muscle sections or myomeres due to storage.

Rating scale:
1 - Intact, no separations.
4 - Some separations but slight, random about fillet and not objectionable.
6 - More advance separation with possible pattern across portions of fillets.
10 - Obvious, unattractive separations with distinct patterns across portions of fillets. Reject.

Texture: Subjective measure of meat firmness and/or resilients based on muscle reaction to finger pressure.

Rating scale:
1 - Firm and resilient returning to original shape.
4 - Delayed return to original shape.
6 - Some deformation and softening.
10 - Distinct deformation, soft tissues, and fragmentation. Reject.

Odor

Rating scale:
1 - Odor absent, to very mild fresh fish aroma.
4 - Fish odor evident, typical, expected.
6 - Fish odor, more objectionable, slight evidence of spoilage by-products.
10 - Distinct spoilage odors, putrid, ammonia-like, reject.

Table A-5. Sensory evaluation sheet for determination of the quality of salmon or mahi-mahi fillet

Fish fillet: Date: No.:

Defect	Rating scale (1-10)					
	A1	A2	A3	A4	A5	A6
General appearance						
Meat color						
Gapping						
Texture						
Odor						
Defect	B1	B2	B3	B4	B5	B6
General appearance						
Meat color						
Gapping						
Texture						
Odor						
Defect	C1	C2	C3	C4	C5	C6
General appearance						
Meat color						
Gapping						
Texture						
Odor						
Defect	D1	D2	D3	D4	D5	D6
General appearance						
Meat color						
Gapping						
Texture						
Odor						
Defect	E1	E2	E3	E4	E5	E6
General appearance						
Meat color						
Gapping						
Texture						
Odor						

Table A-6. Sensory characteristics for judging the quality attributes of fresh, raw tuna fillet

Appearance

Defect degree

None - Attractive, fresh, clean, intact, no gapping fillet; excellent, preferred product
Slight - Fair, acceptable appearance with some noticeable changes due to loss in full body, slight separation, and initial color changes, some dehydration at edges, possible some initial slime.
Moderate - Fair, less appealing yet acceptable but not preferred. More evidence of color change. More slime evident and tacky surface emerging.
Excessive - Reject due to obvious slime, color banding, and loss of appeal.

Meat color

Defect degree

None - Fresh tuna, no discoloration of meat or connective tissues, rich, and attractive.
Slight - Fair, acceptable, yet some initial color changes.
Moderate - Fair, less appealing more obvious loss of color, yet acceptable by average standards.
Excessive - Reject - distinct objectionable color changes, loss of color.

Texture

Subjective measure of meat firmness and/or resilients based on muscle reaction to finger pressure.

Defect degree

None - Firm and resilient returning to original shape.
Slight - Delayed return to original shape.
Moderate - Some deformation and softening.
Excessive - Distinct deformation, soft tissues, and fragmentation. Reject.

Odor

Defect degree

None - Odor absent to very mild fresh fish aroma.
Slight - Fish odor evident, typical, expected.
Moderate - Fish odor, more objectionable, slight evidence of spoilage by-products.
Excessive - Distinct spoilage odors, putrid, ammonia-like, reject.

Table A-7. Sensory evaluation sheet for determination of the quality of tuna fillet

Fish fillet: Date: No.

Defect	Degree	Rating scale	A1	A2	A3	A4	A5
Appearance defects (flesh consistency)	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Discoloration	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Texture	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Odor	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					

Defect	Degree	Rating scale	B1	B2	B3	B4	B5
Appearance defects (flesh consistency)	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Discoloration	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Texture	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Odor	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					

Table A-7. Continued

Fish fillet: Date: No.

Defect	Degree	Rating scale	C1	C2	C3	C4	C5
Appearance defects (flesh consistency)	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Discoloration	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Texture	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Odor	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Defect	Degree	Rating scale	D1	D2	D3	D4	D5
Appearance defects (flesh consistency)	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Discoloration	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Texture	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					
Odor	None	1					
	Slight	2 - 4					
	Moderate	5 - 7					
	Excessive	8 - 10					

Table A-8. Comparison of AOAC, CE, and GC methods for determination of histamine in tuna

Parameter	AOAC method	CE method	GC method
Extraction	Yes	Yes	Yes
Clean-up	Yes	No	Yes
Derivatization	Yes	No	Yes
Injection volume	N/A	8.4 nl	1 µl
Time/sample	20 min	10 min	45 min
Detection limit	10 ppm	5 ppm	10 ppm
Instrument	Fluorometer	CE	GC
Histamine recovery	93.8 %	100.6 %	77.3 %
Equipment cost	$ 4,000	$ 30,000	$ 35,000
# of amines detected	1	1	5
Detector	Fluorescence	UV	FID
Automatic availability	No	Yes	Yes
Solvent used	Methanol	Methanol	Methanol, ethyl acetate, toluene
Intra-assay coefficient of variation (%)	1.6	4.6	10.7
Interassay coefficient of variation	3.1	7.7	17.7

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BIOGRAPHICAL SKETCH

Wen-Xian Du was born on August 16, 1968, in Xiamen, People's Republic of China. She received her bachelor's degree in Food Science and Nutrition from Beijing University in August 1990. After working at the Research and Training Center on Reproductive Medicine for one year as a research assistant, she enrolled at the University of Florida in August 1992. In December 1994, Ms. Du received her Master's degree in the Food Science and Human Nutrition Department. She continued her graduate studies in the same department and is expected to receive her Ph. D. in May 1999.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Cheng-I Wei, Chair
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maurice R. Marshall, Jr.
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sean F. O'Keefe
Associate Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Neal P. Thompson
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John A. Cornell
Professor of Statistics

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1999

Dean, College of Agriculture

Dean, Graduate School